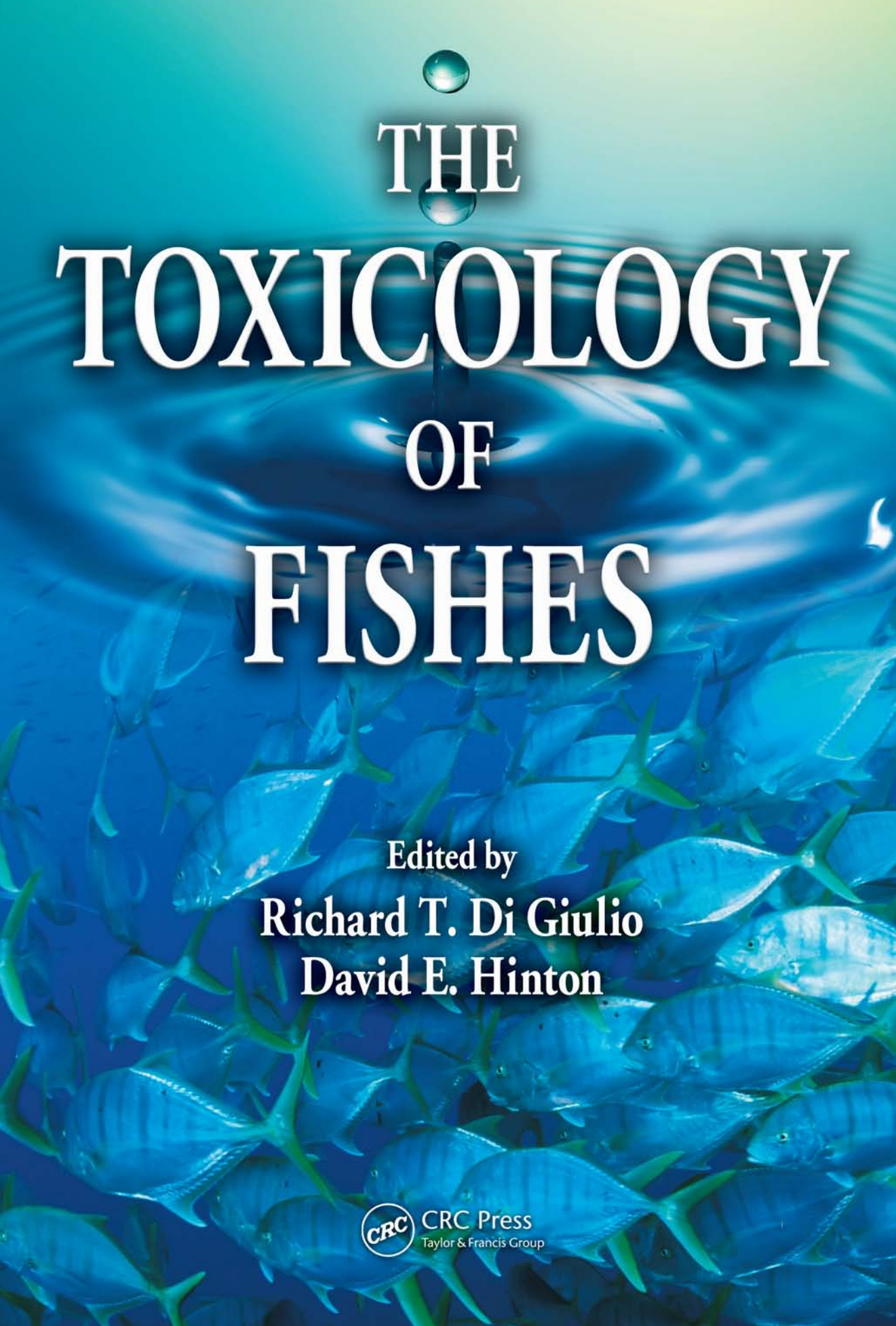
THE
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OF
FISHES
Edited by
Richard T. Di Giulio
David E. Hinton
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Dedication

To my wife, Adriana, and my daughter, Margaret, for your sweetness and kindness, great senses of humor, and love. (RTD)

To my wife, Judith, and to my three daughters, Emily, Jill, and Susan. Your love has shown me a better way. (DEH)

Contents

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Unit I

General Principles

1

Introduction

Richard T. Di Giulio and David E. Hinton

Toxicology, the study of the adverse effects of chemicals, has a rich history, the bulk of which revolves around understanding the effects of poisons on humans. Michael Gallo (2001) provided an excellent and lively historical perspective on human health-oriented toxicology, including historical and literary examples of inquiry from antiquity, the Middle Ages, and the Age of Enlightenment. As he noted, modern toxicology draws upon many fields of basic science; these include molecular biology, chemistry and biochemistry, genetics, organismal biology, ecology, and mathematics. Indeed, the solution to most important toxicological questions requires interdisciplinary approaches drawing on multiple forms of expertise. A distinction that has been used to categorize toxicology is biomedical vs. environmental (or *ecotoxicology*), the former focusing on human health and the latter on free-living flora and fauna and their populations, communities, and supporting ecosystems. The latter is a relatively young field, at least in the sense of a formal science; the term *ecotoxicology* was first used by Truhaut in 1969 (Truhaut, 1977) and is often used to stress the fate and effects of chemicals within an ecosystem (Kendall et al., 2001).

Ecotoxicology has been defined in various ways, but for most of us it means the study of effects that chemical pollutants exert on natural biota. Contributing to the biocomplexity that we are challenged with is the large number of species for which meaningful data are needed. To deal with effects at various levels of biological organization and in a large number of organisms, we must extrapolate findings between species. It should also be noted, however, that some definitions are broadened to include effects on any biosphere component, including humans (Newman and Unger, 2003), and increasing consideration for integrative connections between human health and the well-being of natural systems appears to be enhancing interactions between the fields of biomedical toxicology and ecotoxicology (Di Giulio and Benson, 2002) and also integrations of risk assessments for human health and ecosystem protection (Munns et al., 2003; Suter et al., 2003).

Fish occupy a prominent position in the field of toxicology; they have been employed amply in studies concerning both human and ecological health, probably bridging this divide more than any other class of organisms. Several reasons likely account for this. Fish are by far the most diverse class of vertebrates; the 28,000 species identified to date are greater than the combined numbers for the other classes (Cossins and Crawford, 2005). This taxonomic diversity is reflected in a diversity of body forms, lifestyles, and physiologies, which also reflect the great diversity of aquatic systems that fish inhabit, from freshwater to hypersaline waters, with temperatures ranging from below freezing to >45°C, pressures ranging from 1 to 1000 Atm, and other variabilities in solar radiation, oxygen concentrations, ionic and organic matter compositions, turbulence, bottom environments, and so forth. This species and habitat diversity has long driven the use of fishes for scientific inquiries into the influences of environmental variables on the evolution, genetics, and adaptations of organisms.

In the context of pollution, aquatic systems are highly vulnerable due to their tendency to accumulate relatively high concentrations of chemicals entering from surrounding terrestrial systems, as well from direct inputs; thus, regardless of their source of entry to the environment, aquatic systems are oftentimes repositories for a large array of stressor chemicals (for example, see Chapters 2 and 14 in this volume).

Additionally, aquatic food chains are generally longer than terrestrial food chains; this also likely contributes to observations that many persistent pollutants tend to achieve greater concentrations in aquatic predators, including fishes, compared to terrestrial predators (Clements and Newman, 2002, pp. 300–302). Moreover, aquatic animals are often particularly vulnerable because of elevated exposures arising from their living immersed in the exposure medium (surface water), having highly permeable skin and gills, and other inherent sensitivities. Fish and amphibians, for example, are the only vertebrate groups with anamniotic eggs (lacking a shell or amniotic membrane) and that undergo metamorphosis in surface waters; hence, the embryo–larval stages of these animals are highly sensitive to chemical pollutants (Kendall et al., 2001). These issues likely contribute to lower water quality guidelines to protect aquatic life vs. human health, even though the calculations used to generate such guidelines are more conservative (i.e., protective) in the context of human health (http://www.epa.gov/safewater/mcl.html#mcls).

Fish, not surprisingly, play important roles in setting these water quality guidelines for freshwater and marine systems. U.S. Environmental Protection Agency (EPA) guidelines provide specific recommendations for freshwater and marine species to be used for acute and toxicity tests that are used in establishing these guideline and other chemical safety assessments (U.S. EPA, 1996; see Chapter 15 in this volume). In addition to their role in establishing surface water quality criteria, toxicity tests provide information concerning, for example, relative acute toxicities among various chemicals, relative sensitivities of different species to selected chemicals, and the sublethal effects of chemicals and chemical mixtures. Also, data generated from toxicity tests play important roles in ecological risk assessments (see Chapter 18 in this volume).

In addition to their central role in toxicity testing for ecological effects, fish are perhaps the most employed organisms for biomonitoring. This is likely due in part to the aforementioned propensities of aquatic systems to receive and accumulate environmental contaminants and the diversity and importance of fishes in these systems. Fish-targeted biomonitoring includes a wide variety of approaches for detecting impacts of aquatic contamination, from direct measures of mortality, to broad analyses of population dynamics and community structure, to detection of measures of subcellular change. This last approach, embedded in the term *biomarkers* (see Chapter 16 and the case studies in Unit IV in this volume), has perhaps benefited the most from modern toxicological research with fish models wherein elucidation of the mechanisms of toxic action has received serious consideration and great strides have been made.

In a relatively short time, several decades, aquatic toxicology has moved from a descriptive approach, which was necessary to explore those concentrations of single toxicants within water that were not compatible with the life of individual fishes, to considerations of sublethal concentrations that do not cause death over the short term but do harm the individual, thus making it expend resources to survive in a state of altered equilibrium. Biomarkers of exposure, response, and genetic susceptibility were derived from research in this area, and these helped to cut across questions of bioavailability as the emphasis shifted to host response. Biomarkers illustrate the multiple organ, tissue, and cellular sites of action and the spectrum of responses that were possible. The resultant toolkit, amply illustrated in this volume, is a suite of biomarkers and validated methods that are now used to assess chemical exposures and effects or responses arising from various forms of chronic toxicity.

Again, recent toxicological research with fishes has pursued the study of mechanisms of action. This approach has provided potential tools for ecotoxicologic investigations; however, problems of biocomplexity and issues at higher levels of biological organization remain a challenge. In the 1980s and 1990s and continuing to a lesser extent today, organisms residing in highly contaminated field sites or exposed in the laboratory to calibrated concentrations of individual compounds were carefully analyzed for their responses to priority pollutants. Correlation of biochemical and structural analyses in cultured cells and tissues, as well as *in vivo* exposures, led to the production and application of biomarkers of exposure and effect and to our awareness of important effects such as cancer and endocrine disruption in wild fishes. To gain acceptance of these findings in the greater environmental toxicology community, validation of the model vs. other, better established (often rodent) models was necessary and became a major focus. Resultant biomarkers were applied to heavily contaminated and reference field sites as part of effects assessment and in investigations following large-scale disasters such as oil spills or industrial accidents. It should be noted that the total number of fish species used in mechanistic research and for biomonitoring

is nowhere near the total number of species, and for much of our current information we are dependent on a relatively small fraction of the species.

Through the use of an expanding aquatic toxicology toolkit, effects of stressor chemicals on aquatic organisms are being determined in an integrative manner on individuals and, in some instances, are being extended to population and community levels of biological organization, as well. Most recently, genomic approaches are being explored for their ability to reveal, for example, mechanisms of action, differential sensitivities, and similarities and differences among organisms (see Chapter 5 in this volume). Additionally, they may make substantial contributions to ecological risk assessments (Ankley et al., 2006), as well as promote the integration of human health-oriented and ecologically oriented research and policy (Benson and Di Giulio, 2006).

Why does one need to have a comprehensive treatment of the toxicology of fishes? Particularly with regard to certain aspects of the subject in which enormous and recent growth has taken place, a great deal of new information has been assembled and must be thoroughly reviewed to provide cohesive coverage and to integrate new findings with existing concepts. The next set of challenges that investigators may face in the field will likely cause additional attention to be drawn to organ, tissue, and cellular sites that are being targeted and lead to approaches embodying likely different perspectives.

The Toxicology of Fishes is organized into four units. Unit I, "General Principles," contains Chapters 1 through 6. Following this Introduction, Chapter 2 is devoted to the bioavailability of chemical contaminants in aquatic systems. To students new to this field, bioavailability is critical to our coverage because certain forms of potentially toxic substances in water are bound to particulates in the aquatic medium and are not available for uptake by fish. When uptake has occurred, distribution within the individual fish must be considered, and this is the subject of Chapter 3, "Toxicokinetics in Fishes"; for example, toxicokinetics permits us to understand the distribution within the individual and to approach an improved quantitative estimate of the dose. The fourth chapter is concerned with biotransformations in fishes and, among other things, covers the potential bioactivation of compounds into toxic forms and their conjugation and removal. Chapter 5, "Molecular Mechanisms of Toxicity," provides coverage of the process by toxic states are achieved. Completing this first unit is an additional chapter on mechanisms, particularly those arising through oxidative stress.

Unit II, "Key Target Systems and Organismal Effects," is comprised of Chapters 7 through 13. Chapter 7, "Liver Toxicity," covers the microscopic anatomy of the organ, important aspects of the liver physiology in fishes, and morphological, biochemical, and functional aspects of toxic injury and its consequences. Chapter 8, "The Osmoregulatory System," covers the anatomy and physiology of the gill and its perturbations by metals and other aquatic pollutants; given the extensive surface area and role in uptake of contaminants, the osmoregulatory function of this organ may be compromised by exposure to metals and selected organic compounds. Chapter 9, "Toxic Responses of the Fish Nervous System," provides a description of the central nervous system of fishes and describes a variety of toxic responses, some morphological and others physiological. The coverage leads to improved understanding of the nature of specific toxic induced alterations. Chapter 10, "The Endocrine System," describes the endocrine system of fishes and its toxicity, and receptor-mediated mechanisms and the effect of contaminants on hormone function are covered in detail. Chapter 11 describes the immune system of fish, a known target for certain toxicants that can directly affect the individual's host defense mechanisms. How these toxic responses arise and their significance are the subjects of this chapter. Chemical carcinogenesis of fishes is the subject of Chapter 12, in which a brief history of this interesting aspect of chronic toxicity is provided followed by coverage of molecular aspects of carcinogenesis. Discussions of procarcinogens illustrate important information about fish as models. Both laboratory and field studies are reviewed, and the various fishes that have been studied from contaminated and reference sites are presented. The final chapter in this section, Chapter 13, is a treatment of toxicity resistance; it is important to understand how animals including fishes can adapt to chemical contamination and the long-term consequences of such adaptations.

Unit III, "Methodologies and Applications," is an assemblage of five chapters. Chapter 14, "Exposure Assessment and Modeling in the Aquatic Environment," is followed by a chapter on fish toxicity studies which reviews methods and approaches for determining acute and chronic toxicities in various laboratory applications. Responses that indicate exposure, adverse effects, and genetic susceptibility are included

in Chapter 16, "Biomarkers"; information provided here includes descriptions of a broad array of biomarkers and examples of their application in biomonitoring. In Chapter 17, "Aquatic Ecosystems for Ecotoxicological Research: Considerations and Design Analysis for Fish," the importance of research at higher, ecologically relevant levels of biological organization (populations, communities, and ecosystems) is described, and examples of appropriate designs are provided. Finally, Chapter 18, "Ecological Risk Assessment," deals with the translation of ecotoxicological research into environmental management and policy; the approach of risk assessment considers data on exposure to and toxicity of chemical contaminants, sensitivities of organisms that are likely to be exposed, and the quantitative assessment of risks to aquatic systems.

In Unit IV, "Case Studies," a group of seven chapters provides ample examples of how the principles and approaches presented in earlier units are actually deployed in studies, particularly in the field; for example, Chapter 19 presents an analysis of mining and effects on fish in a Montana river. The study combines chemistry, biological responses, and ecotoxicological findings. In Chapter 20, the effects of synthetic pyrethroid compounds in fish are covered. In Chapter 21, mechanistic insight into the early-life-stage toxicity of certain chemicals is used to assess risks to Great Lakes fish. Both the subject of this field evaluation as well as its use of early life stages are of great interest given recent recommendations by REACH legislation in the European Union to refine, reduce, and reevaluate the use of animals in toxicity testing. Chapter 22 is concerned with the effects of polycyclic aromatic hydrocarbons in fish from Puget Sound, Washington. Chapter 23 examines the effects of the *Exxon Valdez* oil spill on Pacific herring in Prince William Sound. This investigation involved successive years of evaluation and included pathology as a backbone of the field investigations. In Chapter 24 addresses the pulp and paper mill effects studied in streams of Canada; the authors present a consideration of various indicators for the health of surface waters downstream of paper mills. Chapter 25 provides a detailed review of the estrogen-mimicking agents released from treated sewage effluent and their effects on fish inhabiting rivers in England. These case studies illustrate the power of, and absolute need for, highly integrated interdisciplinary research teams to address complex issues of chemical pollution in the aquatic environment.

It is our sincere wish that *The Toxicology of Fishes* will provide a very important teaching tool to introduce new students to the field, and we envision this effort presenting an opportunity for experienced authors and investigators to share their findings and expertise with others. Finally, to prepare a thorough coverage of the toxicology of fishes is a major task and one not completed in a short time due to the multiple roles of those investigators that agree to take on the task of authorship of one or more chapters. To assume this responsibility means that yet another set of tasks was placed on an already busy schedule. To all the participants in this book, we offer our congratulations on a job well done and our sincere appreciation for your efforts on behalf of this endeavor. Due to these efforts we believe this text will become a source of useful information that guides worker and student alike.

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2

Bioavailability of Chemical Contaminants in Aquatic Systems

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CONTENTS

Introduction

Toxicity in fish is the culmination of a series of events involving various physical, chemical, and biological processes (Figure 2.1). Chemicals are released to the environment from different sources; enter aquatic systems in effluents, atmospheric deposition, runoff, and groundwater; and become distributed throughout the water column and underlying sediment. Food organisms for fish become contaminated via contact with water or sediment and via their own food. Fish accumulate chemicals both by ingestion of this contaminated food and by contact of their respiratory surfaces and skin with contaminated water. Accumulated chemical is distributed throughout the fish, and some of this chemical reaches a site of action to elicit toxic effects.

An important aspect of this chain of events is chemical speciation, represented by the pie charts in Figure 2.1. Many chemicals exist in different forms (chemical species) as a result of chemical and biochemical reactions. The identities and relative concentrations of chemical species vary with location and time and differ among the components of an aquatic ecosystem. A fish can be exposed to a mixture of chemical species both in the water it contacts and in the food it ingests. Chemical accumulation and toxicity depend not just on total chemical concentration in the environment but also on how readily the fish can absorb these different chemical species at the gill, across the skin, and within the digestive tract and on how chemical speciation affects distribution throughout the organism. Thus, the chemical will be more or less "bioavailable" to a site of action depending on chemical speciation and various organism attributes.

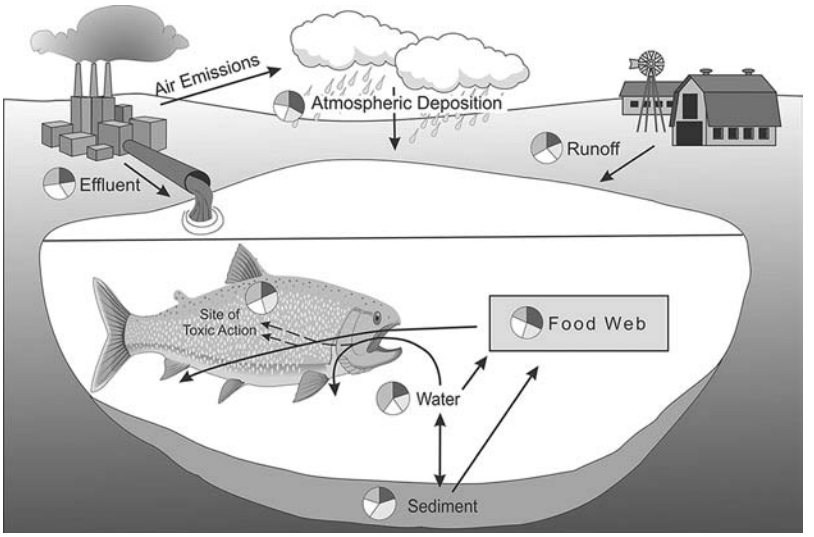

FIGURE 2.1 Chemical inputs to and distribution within aquatic systems, emphasizing accumulation by fish. Pie charts qualitatively represent chemical speciation and how it differs among system compartments.

Definitions of bioavailability vary markedly (National Research Council, 2003) and are often oriented to specific situations; however, these various definitions all address how readily chemicals are accumulated under different circumstances (e.g., for different chemicals, organisms, or environmental conditions). Bioavailability is therefore defined here as *the relative facility with which a chemical is transferred from the environment to a specified location in an organism of interest*. Although it is broad, this definition is still useful because it identifies important factors that must be considered in further refining and applying it for particular assessments:

1. Chemical uptake, and thus bioavailability, depend on certain morphological, physiological, and biochemical attributes of an organism. As such, bioavailability must be defined in terms of a particular type of organism and its physiological state. Caution is needed regarding how well bioavailability relationships for one type of organism can be extrapolated to other types.
2. Bioavailability must be referenced to a specific chemical concentration in the organism of interest. This could be the total chemical in the entire organism, the chemical within a particular tissue, the chemical associated with a specific molecular receptor, or any other measure appropriate to the nature of the toxicity and the goals of the assessment.
3. Bioavailability must also be referenced to a specific environmental concentration. Often this will be the total chemical concentration, with bioavailability being considered an aggregate property of the combined chemical species; however, assessments might also focus on selected subsets of the chemical species or address the comparative bioavailability of individual chemical species. The spatial context of this concentration is also important; often the reference concentration is that in immediate proximity to the organism, but sometimes it covers a large spatial extent. In addition, an exposure time frame might be required because bioavailability relationships can change with time.
4. Transfer pathways of interest must be specified. An assessment might be concerned with only a single route of exposure (e.g., food) or with all possible routes. Depending on how reference concentrations in the organism and the environment are defined, various transport pathways and reactions inside and outside the organism also must be considered.

Whatever the definition and context of bioavailability assessments, their success depends on adequately defining the processes that regulate chemical accumulation. Processes important for determining

bioavailability fall into three categories. First, processes external to the fish determine the concentration and speciation of the chemical to which a fish is exposed. A detailed discussion of fate and transport of chemicals is not in the scope of this chapter, but this topic will receive some attention here because evaluating bioavailability requires some understanding of the nature and origin of the chemical species. Second, the fish can absorb the chemical by various routes and mechanisms that are functions of both chemical speciation and organism physiology. It cannot generally be said that a chemical species is either bioavailable or not; rather, bioavailability is relative and will reflect how much a chemical species contributes, directly or indirectly, to this absorption. Third, once absorbed, the chemical will be modified and distributed within the fish, thus determining the nature and amount of chemical at the site of toxic action.

This chapter is organized into two sections. The first section addresses some general principles of chemical bioavailability with regard to chemical fate in aquatic environments and chemical uptake via different routes of exposure. Additional information regarding chemical uptake and disposition in fish is provided in Chapter 3. This first section also discusses various measures that are used in evaluating bioavailability. The second section presents several case studies regarding the bioavailability of a wide variety of chemicals. These examples are not presented to give a comprehensive review of bioavailability for specific chemicals but rather to illustrate a range of issues and processes that can be important in defining and assessing bioavailability for any chemical.

Assessing Bioavailability: Principles, Processes, and Measures

Chemical Behavior in the Aquatic Environment

Chemicals may enter the aquatic environment via a variety of point and non-point sources, including direct discharges, soil and pavement runoff, and atmospheric deposition (Figure 2.1). Those chemicals that enter aquatic environments in large quantities, that are relatively persistent, or that are very potent typically are of most concern. Some types of anthropogenic chemicals are unlikely to enter the aquatic environment because of how they are used. In other cases, chemicals may not remain in aqueous solution; for example, relatively volatile chemicals usually are not persistent in aquatic systems. In other instances, degradation processes, such as microbial metabolism, aqueous hydrolysis, or photolysis, markedly decrease concentrations of chemicals before or after they enter aquatic systems, thus reducing the probability of significant exposure of aquatic organisms. Approaches exist (based on production volume, use pattern, chemical structure, toxicity, etc.) for predicting the potential hazard of newly manufactured chemicals in aquatic environments. Although consideration of these types of predictive models is beyond the scope of this text, they are generally the first step in determining the need for further application of prospective risk assessment methodologies.

A number of factors related to the structure and properties of a chemical in an aquatic environment will dictate its fate, distribution, and, ultimately, bioavailability to aquatic organisms. Aromatic rings and halogenated substituents, for example, tend to be associated with persistent organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs), as well as organochlorine pesticides such as DDT (and DDE, DDD), dieldrin, and toxaphene. Chemical elements that are neither created nor destroyed but are redistributed in the environment by the activities of humans also are persistent contaminants. Some of the more toxic elements in this class are metals and metalloids such as cadmium, copper, mercury, nickel, zinc, lead, silver, and arsenic. There are also several relatively persistent and toxic organometals such as methylmercury and tributyltin. Even chemicals that are not persistent can be of concern if large quantities are released to aquatic systems or the chemicals are very potent. An example of the former situation would be ammonia, which, under appropriate conditions, can be converted via nitrification to nontoxic products but is a chemical of toxicological significance to fish because of large inputs to aquatic systems. An interesting example of a relatively labile compound that nonetheless remains of concern because of its potency is the synthetic estrogen ethynylestradiol. This component of birth control pills is found only in small concentrations in certain types of municipal effluents, but because of its great affinity for the estrogen receptor it has been associated with an increased incidence of feminized male fish in exposed populations (Desbrow et al., 1998).

Chemical structure and properties dictate not only persistence in the aquatic environment but also the primary compartments (e.g., water, sediment, suspended particles) with which a chemical is associated and, by extension, routes through which a fish is likely to be exposed. One particularly important property controlling the behavior of a chemical in the aquatic environment is its hydrophobicity. A measure of relative hydrophobicity that is often used in aquatic toxicology is the octanol–water partition coefficient (K_{ow}).* Chemicals with large log K_{ow} values, in particular those with log $K_{ow} > 5$, are considered hydrophobic and tend to be associated with organic materials in aquatic systems, especially sediments. Hydrophobic chemicals of environmental concern in this class include most PAHs, PCBs, PCDFs, PCDDs, and some organochlorine pesticides and organometals.

An important variable in chemical fate and bioavailability is the fraction of chemical that is freely dissolved; for example, nonionic organic compounds or cationic metals complexed by dissolved organic carbon (DOC)† are often far less bioavailable than their uncomplexed forms. Although this is an important guiding principle in the area of bioavailability prediction, empirical demonstration of the concept has proved challenging in that it is very difficult to reliably measure freely dissolved chemicals. As an example, although a common operational definition of dissolved metal is the ability to pass through a 0.45-μm filter, it is well known that metals bound to some types of colloidal organic carbon also can pass through this pore size. Analogous problems exist in defining fractions of organic chemicals that are freely dissolved. Often, it is easier to predict than measure dissolved fractions of some chemicals using partitioning and speciation models. As a consequence, assessment of contaminant bioavailability might rely on predictive models that consider properties of both the chemical and the aqueous environment under consideration, in conjunction with measurements of total chemical concentrations. An example of this approach is provided in the case study below concerning 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Another important property used to describe the behavior of many ionizable compounds is their degree of dissociation at pH values typically found in aquatic systems. Charged molecules are more water soluble, while neutral forms of the same chemical tend to be more hydrophobic and thus more associated with organic carbon than water. The speciation of strong acids (or bases) will not be affected by pH variations typical of most aquatic environments; however, the behavior of chemicals with pK_a‡ values in the range of approximately 5 to 9 can be greatly affected by system-specific variations in pH. Chemicals whose pK_a values are important determinants of fate and bioavailability include weak acids, such as phenolic compounds, some surfactants and resin acids, and weak bases such as ammonia.

The interaction of chemicals with biotic and abiotic ligands in the aquatic environment influences their speciation and partitioning into different environmental compartments. A major partitioning phase for many nonionic organic chemicals is DOC and particulate organic carbon (POC), both in the water column and sediments. The interaction between these types of chemicals and organic carbon can be modeled based on their K_{ow} values. A predictable and generally linear relationship exists between the log K_{ow} of a chemical and the log K_{oc},§ so knowledge of K_{ow} can be useful for deriving organic carbon partitioning relationships for predicting bioavailability. In the case of organic and inorganic ions, ionic constituents of the water can affect chemical speciation; for example, certain types of DOC (i.e., humic acids) as well as some inorganic anions can strongly complex cationic metals, thereby controlling partitioning and subsequent bioavailability.

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* The octanol–water partition coefficient (K_{ow}) is the equilibrium ratio of the concentration of a chemical in *n*-octanol to its concentration in water, commonly expressed as its base 10 logarithm, log K_{ow}.

† Dissolved organic carbon (DOC) is the carbon present in the complex mixture of organic molecules dissolved in water, consisting largely of organic acids originating from the decomposition of plant material, including fulvic and humic acids. This is in contrast to particulate organic carbon (POC), which refers to the carbon present in the complex mixture of particulate material suspended in water, including resuspended sediment and detritis, soil particles, leaf litter, bacteria, phytoplankton, and zooplankton. POC and DOC are often defined operationally as that portion of the total organic carbon (TOC) in water retained and not retained, respectively, by a filter with a pore size of 1.0 μm or less (commonly 0.45 μm).

‡ The acid dissociation constant (K_a) is the equilibrium constant for the dissociation of an acid into hydrogen ion and its conjugate base. $pK_a = -\log_{10}(K_a)$ and is equal to the pH at which half of the chemical is ionized.

§ The organic carbon–water partition coefficient (K_{oc}) is the equilibrium ratio of the concentration of a chemical associated with organic carbon (measured as the mass of chemical per mass of carbon) to its free concentration in water. The value for this coefficient will depend on the composition of the organic carbon phase, which varies among aquatic systems, between the water column and sediment, and between dissolved and particulate phases.

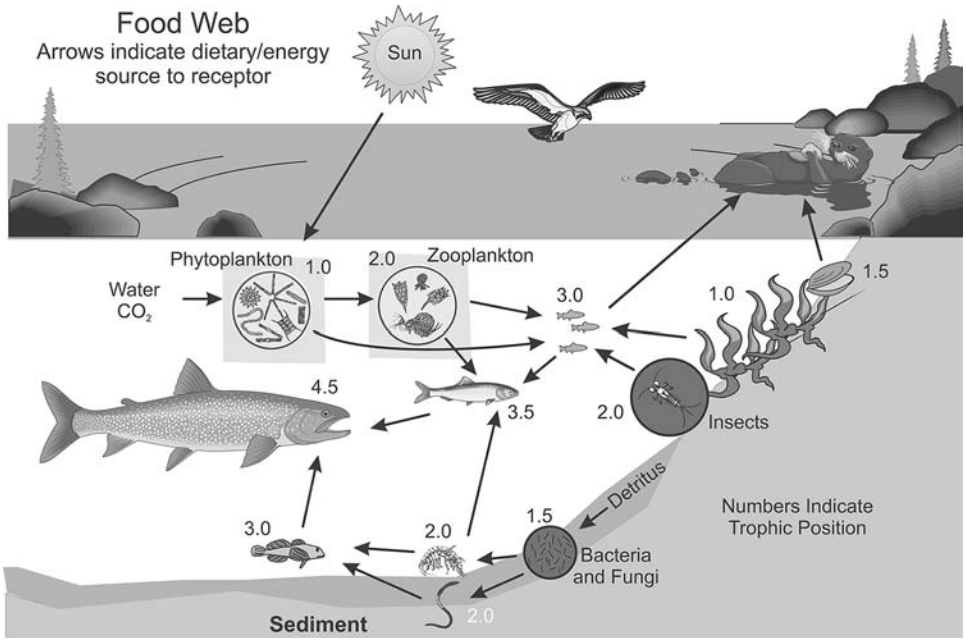

FIGURE 2.2 Movement of chemicals through aquatic food webs. Numbers denote trophic level of organism (e.g., 1.0, primary producers; 2.0, strict herbivores).

An additional type of interaction that can be important for the speciation of ionized chemicals is competition with other ions in speciation reactions. This is particularly important for divalent cationic metals, such as copper (see case study below), whose speciation and partitioning can be greatly influenced by structurally similar but relatively nontoxic cations such as calcium and magnesium.

An understanding of chemical bioavailability requires understanding not only the chemical reactions within an environmental compartment but also how the chemical moves among compartments. An important route of exposure for cationic metals, for example, is via the gill in fish, yet the major repository of some metals in many aquatic systems is the sediment. Therefore, to completely assess the potential for a cationic metal to produce toxicity requires an understanding of bioavailability as it relates to both water and sediment. In fact, the dynamic relationship between the water column and sediments, which serve both as a source and sink for contaminants, is so critical that most state-of-the-art fate and effects modeling at the watershed level explicitly considers interactions between the two compartments.

The interplay between the water column and sediments becomes particularly important for food webs that determine contaminant exposure in fish diets (Figure 2.2). Contaminants in these food webs can originate from the water column via absorption from solution by phytoplankton and other suspended particles, which are consumed by filter feeding animals, which in turn support a series of predators. The contaminants might also originate from sediments, where various invertebrates accumulate chemicals from pore water or ingested sediment particles, thus providing another food base for predators. The resulting dietary exposures to fish will have both water column and benthic components, the relative importance of which will vary depending on the distribution of contaminant between the water column and sediment, chemical speciation within the water and sediment, the nature of the food web, and the position of the fish within the food web.

Accumulation via Gills and Skin

Fish gills serve a variety of physiological functions, including respiratory gas exchange, osmoregulation, nitrogen excretion, and control of acid–base balance (Hoar and Randall, 1984). Because of these functions, fish gills have the following features important for the exchange of toxic chemicals between a fish and its environment:

1. They process large volumes of water, ranging from several to hundreds of times the volume of the fish per hour, and are supplied by a large blood flow (typically about an order of magnitude lower than the water flow).
2. They have a large surface area with a small distance separating water and blood. To accomplish this, gills contain a grid of numerous small, parallel, plate-like structures called *secondary lamellae*. Each pair of lamellae delimits a narrow channel through which water flows, and each lamella contains a blood space separated from the water by a thin layer of epithelial tissue.
3. Within the gill lamellar system, blood and water generally flow counter-current to each other, which improves the efficiency of transfer of material between the two. This combination of counter-current exchange, large surface area, and short diffusion distance results in highly efficient extraction of the small concentrations of oxygen present in water. This high extraction efficiency also depends on oxygen being (a) a small, neutral molecule that readily diffuses across lipid cellular membranes, and (b) strongly bound to hemoglobin in blood (which helps to maintain steep diffusion gradients between water and blood as they flow through the gill).
4. Gill epithelial tissue contains a variety of biochemical systems that regulate or otherwise affect the exchange of various chemicals between blood and water passing through the gills. Sites on the cellular membranes for ion exchange (transport proteins, ion channels) can serve as uptake sites for toxic chemicals with suitable physicochemical properties.
5. Because of the chemical exchanges that are part of their normal functions, gills create a chemical environment adjacent to their surface that can differ markedly from that of the surrounding water. Due to excretion of carbon dioxide and ammonia, the pH at the gill surface of a fish can differ from that in the surrounding water (Lloyd and Herbert, 1960; Playle and Wood, 1989; Wright et al., 1991). Gill epithelial cells are also covered by a thin layer of polysaccharide mucus, which can affect chemical speciation at the gill surface and in the adjacent water (Tao et al., 2002).

Figure 2.3 illustrates transport pathways and chemical reactions that can be important for the uptake of toxic chemicals at fish gills. For simplicity, just two species of a toxic chemical (T) are shown here—that which is bound to some other chemical constituent (B) in water or blood and that which is not bound (free). These two species are connected by a double arrow to represent the speciation reaction by which they interconvert. Sometimes speciation reactions are slow enough that chemical species will not change during passage through the gill; however, many reactions are fast enough that chemical species can be altered within the gill, with possible consequences for uptake rates and bioavailability.

As the toxic chemical is swept along the water channel, free chemical can be absorbed into the epithelial tissue via various routes and mechanisms. If the chemical can readily cross lipid cellular membranes, a principal route of uptake will be passive diffusion across the epithelial tissue (arrow 1 on Figure 2.3). Other chemicals might have the appropriate size and electrochemical properties to be taken up via specific biochemical exchange sites on the epithelial tissue surface (arrow 2 and rectangular S). A significant site for ion loss in freshwater fish can be via junctions between epithelial cells. These junctions are not depicted on Figure 2.3 but might also be important for the uptake of chemicals that are not effectively absorbed via other routes.

The bound chemical might also be directly absorbed via one or more of the same routes as the free chemical, although probably at a different rate (arrow 3); however, the bound chemical can also affect uptake in other ways. If absorption of the free chemical significantly reduces its concentration within the lamellar channel and if the speciation reaction is fast enough, net dissociation of the bound chemical (arrow 4) will replenish the free chemical concentration, supporting additional uptake. In this way, the bound chemical can be considered bioavailable, even though it is not directly absorbed. Such shifts in speciation equilibrium require significant net absorption of the chemical during passage through the gill, and so will be important only when chemical concentrations in fish are below those in equilibrium with the surrounding water. This will be true during the early stages of exposure to a chemical but can also be true at steady state if the chemical is rapidly metabolized or eliminated via routes other than the gill.

These uptake relationships can be modified by the chemical characteristics of the water in the gill lamellar channels (C in Figure 2.3). This chemistry is a function of both the incoming exposure water

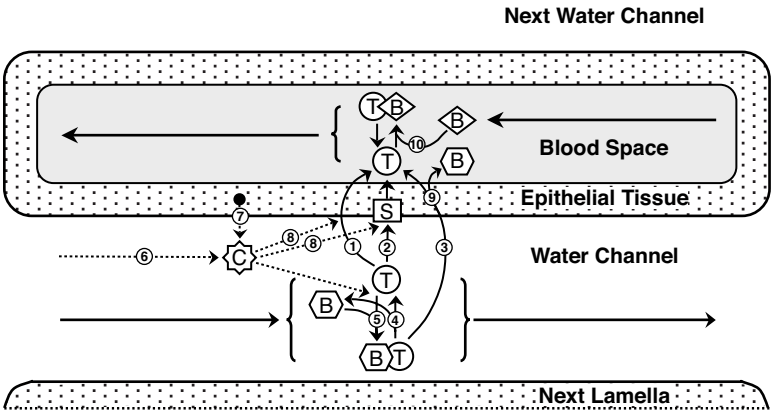

FIGURE 2.3 Conceptual model of contaminant uptake at fish gills. Diagram depicts a secondary lamella of a fish gill as epithelial tissue enclosing a space through which blood flows and shows an adjacent lamellar channel through which water flows. See text for explanation of symbols.

(dotted arrow 6) and various chemical exchanges at the gill surface (dotted arrows). Changes in this chemistry can shift the speciation of the toxic chemical, causing net increases or decreases in the amount of free chemical (arrows 4 and 5, respectively). Chemical characteristics of the exposure water can also affect uptake rates across the gill epithelium by affecting cellular membrane characteristics (dotted arrows marked 8); for example, certain chemical constituents might compete with the toxic chemical at, or otherwise modify the properties of, the exchange site.

After the chemical has been absorbed into and across the epithelium, its speciation can also change. Toxic chemical that is absorbed while bound to other chemicals will dissociate in the chemical environment of the organism (forked arrow marked 9), unless the dissociation reaction rate is slow or the same binding agent exists at similar concentrations within the organism. More importantly, the speciation of the toxic chemical can be affected by a variety of chemical constituents within the organism (arrow 10). These various reactions will alter chemical gradients and thus affect uptake, just as binding to hemoglobin helps maintain high uptake rates of oxygen. If these speciation reactions are rapid enough, each chemical species in the exposure water that is absorbed will contribute to the internal concentrations of *all* species and thus to the effective dose, in proportion to the rate at which it is taken up; however, in cases where speciation reactions are slow, toxic chemical species inside the fish may retain a "memory" of their identity outside the fish, such that different species may not contribute to toxicity to the same degree as they do total accumulation.

Various processes identified in Figure 2.3 are reflected in data from McKim et al. (1985), who measured the uptake of 14 organic chemicals across the gills of large rainbow trout (*Oncorhynchus mykiss*). For neutral organic compounds of moderate hydrophobicity ($3 < \log K_{ow} < 6$), chemical extraction efficiencies (i.e., the fraction of chemical removed from water flowing into the gill) were similar to that of oxygen. Efficiencies were high for these chemicals because, like oxygen: (1) they exist in exposure water almost entirely as uncomplexed, small, neutral molecules that can readily diffuse into and across the gill epithelium, and (2) they bind to certain components in the blood, thereby maintaining a strong diffusion gradient from water to blood. Accumulation rates for these chemicals are primarily limited by the rate at which water is pumped through the lamellar water channels.

Less efficient uptake occurred for chemicals with either lower (<3) or higher (>6) log K_{ow} values and for those chemicals that were partially ionized. These lower uptake efficiencies can be understood in terms of chemical speciation and membrane transport properties. For chemicals with $\log K_{ow} < 3$, uptake is slower because these chemicals do not bind as strongly to blood components, which results in the free chemical concentrations in the blood increasing significantly during passage through the gill, thereby reducing diffusion gradients. The accumulation rate of such chemicals is primarily limited by the rate at which blood flows through the secondary lamellae, in contrast to the water-flow-limited chemicals discussed above. Chemicals with $\log K_{ow} > 6$ will diffuse less readily across cellular membranes, either

because the larger size of these molecules reduces their ability to penetrate lipid membranes (Opperhuizen et al., 1985) or because their high hydrophobicity results in them being bound to even larger organic substances in the exposure water (Black and McCarthy, 1988). For the partially ionized chemicals, lower uptake rates occur because the charged forms diffuse less readily across lipid membranes. For both of these latter two groups of chemicals, accumulation rates are limited primarily by their diffusion across the epithelium rather than by water and blood flows.

Uptake of toxic chemicals from water via the skin will generally be much less important than uptake via the gills because the skin of fish typically provides less surface area, a thicker and less permeable diffusion barrier, slower transport of water to the exchange surface, less blood flow, and no countercurrent flow of water and blood. McKim et al. (1996) reported uptake of chlorinated ethanes via skin to be only a few percent of total uptake in adult rainbow trout and channel catfish (*Ictalurus punctatus*). For smaller fish, however, uptake via skin can be important (Lien et al., 1994; Saarikoski et al., 1986), because, as size decreases, skin is generally more permeable and has an increasing surface area relative to the gills. This is especially true for fish embryos and larvae, whose gills are not well developed and for which respiration can be largely via their more permeable skin. Although many of the same general principles apply whether uptake is via skin or gill, details regarding epithelial permeabilities, transport mechanisms, and the chemical microenvironment differ between gill and skin and are not well characterized. The relative importance of gill vs. skin generally is not known or even explicitly considered in chemical risk assessments, but it should be remembered that there will be a skin component of uptake of waterborne chemicals that could be significant and might exhibit bioavailability relationships different from the gill.

Accumulation via Diet

When chemicals in water partition strongly into solid phases or exist as dissolved forms that are poorly absorbed at gill and skin surfaces, the major route of exposure to fish can be via ingestion of contaminated food (or sediment for some species) and subsequent absorption within the gastrointestinal tract (GIT). The contents of the GIT represent an extension of the external environment, albeit one that is substantially modified by the process of digestion. Structural and functional features of the GIT that contribute to dietary uptake of xenobiotic chemicals were reviewed by Kleinow and James (2001). Briefly, these features include:

1. The large absorptive surface area is increased in many species by the presence of blind diverticula called *pyloric ceca* (Buddington and Diamond, 1987).
2. One or a few epithelial cell layers separate the contents of the gut lumen from elements of the blood circulation.
3. A high degree of tissue vascularization is present; blood perfusion of the GIT increases following the consumption of a meal and is probably controlled at a regional level so blood flow is directed to gut segments where digestion is occurring (Axelsson et al., 2000).
4. The contact time between gut contents and the gut epithelium is relatively long; total gut transit times in fish vary widely but generally range from a few hours to a day or more.
5. Ingested food items are broken down by the combined effects of acidification, enzymatic action, and physical disruption, releasing chemical contaminants to the liquid environment within the GIT. The rate of digestion and efficiency of nutrient uptake depend in turn on factors such as temperature, feeding frequency, meal size, and food digestibility.

The processes that control chemical flux between gut contents and the general circulation are not as well understood as those occurring at the gills. In general, compounds for which dietary uptake is an important route of uptake tend to remain associated with components of the meal until these components are taken up by the fish. Mixed micelles resulting from the digestion of dietary lipid sequester lipophilic organic compounds. These micelles are subsequently broken down at the surface of intestinal epithelium, releasing their contents within the aqueous boundary layer. Metals that possess high affinity for protein

thiol groups tend to complex with amino acids and small peptides. Specific transport systems may exist to move these complexes across the gut epithelium, thereby facilitating metal uptake. Simple diffusion across the gastrointestinal epithelium may control the rate of uptake of some compounds. Uptake of poorly diffusing chemicals may occur due to pinocytosis of proteins and associated bulk media (McLean and Donaldson, 1990).

When used in the context of dietary uptake, the term *bioavailability* generally refers to the fraction of chemical that is absorbed by an animal following the ingestion of a contaminated meal. *Dietary absorption efficiency* may in theory be determined by measuring the difference in chemical mass in food and feces (Penry, 1998). In practice, however, such measurements are difficult to make. More commonly, researchers estimate absorption efficiency by measuring the amount of chemical retained by a fish after feeding it a defined ration. This value is referred to as the *dietary assimilation efficiency* and represents the net result of absorption and elimination, including biotransformation. To the extent that elimination occurs during the course of such an experiment, dietary assimilation efficiency will be lower than true absorption efficiency. For this reason, feeding studies designed to estimate absorption efficiency based on accumulated chemical residues are most useful when the compound is eliminated from the animal very slowly. In prolonged feeding studies, dietary assimilation efficiency is expected to decline as fish accumulate chemical and approach a dynamic steady state.

Alternatively, researchers have used simple kinetic models and independently obtained estimates of elimination rate (generally from depuration studies) to estimate dietary absorption efficiency (Bruggeman et al., 1981; Niimi and Oliver, 1988). This method also relies on the use of measured whole-body concentration data. By using a kinetic model, however, it is possible to account for the effect of chemical elimination. The absorption efficiency constant determined in this manner does not change as fish accumulate chemical; instead, the fish's approach to steady state is determined by the balance between uptake and elimination processes. A portion of the chemical eliminated by fish may be contained in feces, but the contribution of these losses to total elimination is unknown. Using this approach, fitted absorption efficiency constants may be subject to error when the elimination rate is very low and therefore difficult to estimate.

Another way to characterize dietary uptake is to employ methods developed in pharmacokinetic studies with mammals to estimate the *oral bioavailability* of drugs. In this procedure, the compound of interest is administered in food, and samples are collected to characterize plasma concentrations of the chemical over time. Later, after the first dose has been cleared, the animal is administered an equivalent amount of compound as either an intravascular (i.v.) or intraperitoneal (i.p.) dose, which is considered to be 100% bioavailable. Oral bioavailability is then calculated as the ratio of the area under the plasma concentration–time curve (AUC) for oral dosing to the AUC for i.v. or i.p. dosing. A variation on this method involves the use of two groups of animals, one of which is dosed i.v. or i.p. and the other fed a contaminated diet. Oral bioavailability determined in this manner may be thought of as the fraction of the ingested dose that is absorbed across the GIT and enters the systemic circulation.

Feeding studies with fish have provided empirical dietary uptake data for a large number of organic compounds as well as some metals. Based on a review of literature values for organic compounds, Gobas et al. (1988) reported a dependence of absorption efficiency on chemical log K_{ow}; absorption efficiencies averaged about 50% for chemicals with log K_{ow} values between 4 and 7 and then declined progressively at higher log K_{ow} values. Other researchers have reported a lack of dependence of assimilation efficiency on chemical log K_{ow} (Burreau et al., 1997). Dietary uptake of some metals by fish appears to be regulated, resulting in a less-than-proportional increase in whole-body concentration for a given increase in the trace element concentration in food. This phenomenon has been observed for both essential (e.g., zinc) and nonessential (e.g., cadmium) metals (Douben, 1989; Spry et al., 1988). The mechanisms by which this regulation is accomplished remain poorly understood and could conceivably involve concentration-dependent changes in absorption across the gut, although adaptive changes in elimination pathways are also likely (Reinfelder et al., 1998).

Variability in estimated absorption efficiency values for fish may also be due in part to methodological considerations. Feeding studies with fish are generally conducted by spiking test chemicals into prepared diets. Less commonly, an effort is made to incorporate chemicals into live prey items. Limited data suggest that organic chemicals incorporated into live prey are taken up more efficiently than the same

compounds spiked into synthetic diets or administered in oil (Burreau et al., 1997; Nichols et al., 2001). Comparisons of metal uptake from formulated and natural diets have yielded varying results (Clearwater et al., 2002).

Vetter et al. (1985) used autoradiographic methods to show that [^{14}C]-benzo(*a*)pyrene remains associated with lipid throughout lipolysis, lipid absorption, and the formation of intracellular fat droplets in the gut epithelium. These observations underscore the close association between hydrophobic compounds and lipids throughout digestion but provide little information on processes that actually limit the rate of absorption at the gastrointestinal epithelium. Working with goldfish, Gobas et al. (1993a) found that dietary uptake of some very hydrophobic compounds ($\log K_{ow} > 6.3$) declined with an increase in dietary lipid content, while uptake of some moderate to high $\log K_{ow}$ compounds ($4.5 < \log K_{ow} < 6.3$) did not vary among treatment groups. These findings were interpreted as evidence that simple diffusion controls the rate of uptake across the gut epithelium.

Using an *in situ* channel catfish intestinal preparation, Doi et al. (2000) showed that the bioavailability of [^{14}C]-3,3′,4,4′-tetrachlorobiphenyl ([^{14}C]-PCB 77) varied with the fatty acid composition of lipid micelles and that these differences were related to the ability of micelles to solubilize the compound. These observations suggest that dietary absorption of hydrophobic compounds depends on the capacity of lipid micelles to deliver chemical to the gastrointestinal epithelium and possibly on regional differences in fatty acid absorption. In addition, Doi et al. (2000) found that dietary pretreatment of fish with unlabeled PCB 77 reduced the bioavailability of a subsequent radiolabeled dose. This decrease in uptake efficiency was accompanied by lower concentrations of [^{14}C]-PCB in the cytosolic fraction of gut tissues. The mechanism responsible for this pretreatment effect is unclear but may have been due to unlabeled PCB occupying binding sites on proteins and lipids that transport chemicals through the cytosol.

To the extent that simple diffusion plays a role in controlling chemical uptake within the gut, the process of digestion will tend to promote uptake of hydrophobic compounds by increasing the chemical activity gradient between the gut contents and blood (Connolly and Pederson, 1988; Gobas et al., 1993b; Nichols et al., 2004). This happens for two reasons: (1) a reduction in meal volume increases the concentration of chemical remaining in the GIT, and (2) absorption of dietary lipid substantially reduces chemical affinity for material remaining within the GIT and at the same time causes a transient increase in chemical affinity for the absorbing tissues. Together, these two outcomes of digestion create a potential for the lipid-normalized chemical concentration in a predator to exceed that of its prey, a condition referred to as *biomagnification*. Initially, this finding might appear to violate thermodynamic principles but, in fact, does not if equilibria are evaluated relative to digested rather than ingested material.

Chemical uptake from dietary sources may be reduced by biotransformations mediated by gut microflora or occurring within the gastrointestinal epithelium. In studies with *in situ* gut preparations, the GIT has been shown to play an important role in the metabolism of PAHs by fish, altering their form and limiting transport to the systemic circulation (Kleinow et al., 1998; Van Veld et al., 1988). Operating in series with first-pass metabolism in the liver, this activity can substantially reduce the accumulation of contaminant residues by fish (James and Kleinow, 1994; Kleinow and James, 2001; Van Veld, 1990).

In an environmental setting, chemical uptake by an organism from its diet also depends on the accumulation relationships for the organisms that it consumes (Figure 2.2). Under these circumstances, it becomes necessary to extend bioavailability concepts to an entire assemblage of species. Organisms that occupy the base of an aquatic food web generally accumulate chemicals directly from water. For these animals, chemical speciation and the microenvironment within which the animal lives may be critical determinants of uptake. Chemicals that possess characteristics that favor dietary uptake will then pass through a series of trophic transfers, ultimately accumulating in fish that occupy the highest trophic level. At each step along the way, the chemical concentration in the organism represents a dynamic balance between uptake and elimination processes. Organism growth rate will also influence the concentration of slowly accumulating chemicals by determining the mass of tissue into which chemicals are distributed.

An extension of bioavailability concepts from individual animals to entire food webs must also take into account species differences in biotransformation; for example, some organic chemicals are efficiently metabolized by benthic invertebrates. In extreme cases, this metabolism proceeds to such an extent that organisms at higher trophic levels are exposed only to metabolites. Other organic chemicals may accumulate at lower trophic levels but may be absent or present at reduced concentrations in fish tissues

because of preferential metabolism by vertebrates (see benzo(*a*)pyrene case study below). Similar considerations influence the ecosystem behavior of trace elements. With few exceptions (notably mercury as methylmercury and selenium as selenomethionine), concentrations of trace elements tend to decrease with increasing trophic level due to more efficient regulation by animals at higher tropic levels (Reinfelder et al., 1998). The overall effect of metabolism (or trace element regulation) on chemical exposure to higher trophic level organisms is one of the most difficult aspects of bioavailability to predict because of uncertainties associated with the metabolic capabilities of different species and life stages, as well as differences in food web structure and function.

Finally, a potential source of complexity in describing and predicting chemical accumulation by fish at high trophic levels exists when chemical concentrations in water and sediments are not in thermodynamic equilibrium with one another. This condition arises when the rate of chemical exchange between water and sediment is slower than the rate of change of chemical inputs to the system. The degree of disequilibrium between sediments and water is an important ecosystem characteristic that influences the relative contributions of benthic and pelagic food chains to bioaccumulation in higher trophic level aquatic organisms. A key element in assessing bioavailability in the presence of such disequilibria are food-web models that can address the relative contribution of sediment and water-column contamination to chemical accumulation in fish (Burkhard et al., 2003).

Measures Used in Assessing Bioavailability

Although bioavailability reflects various complexities, as discussed above and in the case studies below, it still is primarily the simple comparison of the amount of chemical accumulated by an organism to the amount of chemical the organism is exposed to. As such, a bioavailability assessment generally addresses the ratio of a measure of accumulation to a measure of exposure and how this ratio varies among exposure conditions, organisms, and chemicals. Various measures that are used in bioavailability assessments are addressed in this section.

In some cases, an absolute amount of chemical can be specified for the exposure an organism receives. This is the case in studies of dietary bioavailability when test organisms ingest a known amount of contaminated food, and bioavailability can be examined in terms of the fraction of this dose that is accumulated. As discussed in the dietary section above, measures of this include the *dietary absorption efficiency*, the fraction of the total amount of a compound consumed by an animal that is absorbed across the gastrointestinal epithelium, and the *dietary assimilation efficiency*, the fraction of the total amount of a compound consumed by an animal that is retained in body tissues. An analogous absolute measure, *chemical extraction efficiency*, can be determined for chemical uptake at fish gills using systems in which water flow and chemical concentration changes across gills can be directly measured (McKim et al., 1985).

More often, chemical exposure is measured in terms of environmental concentrations, not in terms of absolute amounts processed by the organism. When the environmental concentration is that in water, measures of accumulation often used are the *bioconcentration factor* (BCF) and *bioaccumulation factor* (BAF), both of which equal the ratio of the concentration of a substance in the tissue of an aquatic organism to its concentration in the exposure water. These measures differ in that *bioconcentration* refers just to uptake directly from exposure water (via gills, skin, ingestion of water), whereas *bioaccumulation* refers to uptake via all exposure routes, including food and sediments as well as water.

Defining a BCF or BAF requires specification of the chemical concentration of interest in both the organism and the exposure water. For the accumulated chemical, the concentration might be that in the entire organism or in a specific tissue and might be calculated on a wet or dry weight basis. For organic chemicals that partition strongly to lipid components in an organism, the concentration might also be calculated as the mass of chemical in the organism or tissue divided by the weight of lipids (i.e., lipid-normalized), rather than the total weight. Such a measure is more reflective of chemical activity and can provide more meaningful comparisons among organisms with different lipid contents. For the exposure water, the concentration might be the total chemical in a volume of water, or that which is dissolved or freely dissolved (that portion of the dissolved chemical that is free of any associations with other solutes). BCFs and BAFs also require specification of a time frame. They might be steady-state values that would result from long exposures or might refer to accumulation over some specified shorter time frame.

Measures analogous to the BAF can also be defined when exposure is referenced to chemical concentrations in sediment or food. For example, the *biota-sediment accumulation factor* (BSAF) was developed for assessments of hydrophobic chemicals for which concentrations in the water column are difficult or impossible to adequately measure and for which sediment-based food chains are an important route of exposure (Ankley et al., 1992). This factor is defined as the ratio of a lipid-normalized chemical concentration in an aquatic organism to the organic carbon-normalized chemical concentration in the surface sediment. Organic carbon-normalized sediment concentrations are calculated relative to the weight of organic carbon in the sediment rather than the total weight. Like lipid normalization, this provides a measurement that is better related to chemical activity and is more useful for comparing and predicting accumulation of hydrophobic organic chemicals among different sites and exposure conditions.

As discussed in the previous section on dietary uptake, digestive processes can increase the activity of chemical within the gastrointestinal tract, resulting in higher concentrations in an organism than in the food it consumes (i.e., biomagnification). The *biomagnification factor* (BMF) is the unitless factor by which the concentration of a substance in an organism at one trophic level exceeds the concentration in organisms that occupy the next lower trophic level. For organisms at higher tropic levels whose chemical exposure is primarily via diet, biomagnification can be of great importance for determining their exposure concentrations. For such organisms, the bioavailability of a chemical relative to its environmental concentration will be a function of a set of accumulation relationships, including BMFs, for various organisms throughout the food web.

Chemical uptake rate constants (the ratio of the uptake rate to the exposure concentration) can also serve as useful measures in bioavailability assessments. Such coefficients are proportional to a BCF or BAF for short exposures in which chemical elimination is small compared to uptake. For longer exposures, these coefficients will also be proportional to the BCF or BAF, provided the elimination rate constants do not vary significantly across the different exposure conditions for which bioavailability is being assessed. If elimination rates do vary, then uptake rates would not be a good overall measure for assessing bioavailability but can still be useful for investigating some of the processes regulating bioavailability.

Sometimes chemical accumulation by an organism is not, or cannot be, adequately characterized; however, if a certain level of toxicity over a specified exposure period can be assumed to reflect a fixed amount of chemical accumulation, then exposure concentrations causing this level of toxicity can serve as a measure of bioavailability. By the relationship BAF = (accumulated concentration)/(exposure concentration), the exposure concentration is inversely proportional to the BAF if the accumulated concentration is constant. The inverse of a toxic effect concentration therefore can serve as a surrogate for the BAF. The case studies below for ammonia and copper will illustrate this, using 96-hour LC_{50} values (the concentration causing 50% lethality over a 96-hour exposure) as measures of how bioavailability is affected by exposure conditions.

Assessing Bioavailability: Case Studies

To further illustrate the general principles and processes discussed above and to introduce approaches for assessing and describing bioavailability, this section considers certain aspects of the bioavailability of selected chemicals with different physicochemical characteristics. Included in these case studies are (1) an ionizable inorganic compound (ammonia), (2) ionizable organic chemicals (phenols), (3) cationic metals (copper), (4) organometals (mercury), and (5) nonionic organics with differing properties (2,3,7,8-tetrachlorodibenzo-*p*-dioxin and benzo(*a*)pyrene). Although these examples address specific chemicals, they are intended to exemplify concepts of bioavailability that are more generally applicable.

Ionizable Inorganic: Ammonia

Aquatic systems can have ammonia concentrations high enough to adversely impact fish as a result of wastewater treatment plant discharges, degradation of nitrogen-containing organic matter, fertilizer runoff, and industrial sources. The two major chemical species of ammonia are weakly basic un-ionized

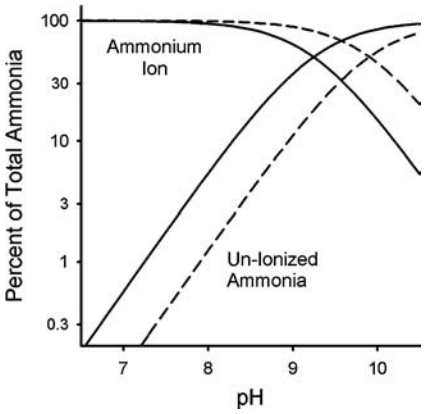

FIGURE 2.4 Effects of pH on the relative abundance of un-ionized ammonia vs. ammonium ion at 5°C (dashed lines) and 25°C (solid lines).

ammonia (NH_3) and its conjugate acid, ammonium ion (NH_4^+), which are in equilibrium with each other according to the following expression:

$$K_a = \frac{[H^+][NH_3]}{[NH_4^+]} \tag{2.1}$$

The acid dissociation coefficient (K_a) for this reaction ranges from approximately 10^{-10} at 0°C to 10^{-9} at 30°C (Emerson et al., 1975). The relative amounts of these species, therefore, can vary markedly across the ranges of pH and temperature found in natural aquatic systems (Figure 2.4).

Ammonia toxicity has been found to increase greatly with increasing pH for various fish species (see Figure 2.5A). This increased toxicity is correlated with large increases in the fraction of total ammonia that is un-ionized; therefore, un-ionized ammonia is presumably responsible for this increased toxicity and, because it is present at much lower concentrations, is apparently much more bioavailable than the ammonium ion. This has not been directly demonstrated based on uptake of ammonia at fish gills, but un-ionized ammonia is considered to be a primary component of ammonia excretion, despite its low concentration relative to ammonium ion, because its lack of charge and smaller size result in a higher permeability than ammonium ion across biological membranes (Wood, 1993).

When a single chemical species is the predominant bioavailable form, it can be useful to express toxicity on the basis of just that species. This results in relationships that are more constant relative to environmental variables and can better demonstrate the role speciation has in toxicity. Ammonia toxicity is therefore often reported on the basis of un-ionized ammonia. When the LC_{50} values in Figure 2.5A are recalculated based just on the concentration of un-ionized ammonia (Figure 2.5B), the overall variation of LC_{50} values is reduced, and the LC_{50} values are roughly constant for pH = 7.8 to 9.0. Such constancy of the LC_{50} values suggests that un-ionized ammonia is the predominant source of toxicity for this pH range, despite it being present at much lower concentrations than ammonium ion.

At near-neutral and acidic pH values, however, toxicity on the basis of un-ionized ammonia is not constant, but rather increases with decreasing pH (Figure 2.5B). This suggests that ammonium ion has enough bioavailability to cause it to be the predominant source of toxicity when concentrations of un-ionized ammonia are extremely low. Some bioavailability of ammonium ion is expected based on studies of ammonia excretion, which have demonstrated that ammonium ion can be excreted at fish gills by diffusion along an electrochemical gradient from blood to water and by carrier-mediated exchange with sodium or hydrogen ion (Wood, 1993).

These data qualitatively suggest that ammonia toxicity is a joint function of both un-ionized ammonia and ammonium ion. A quantitative description of this relationship can be derived as follows. If the uptake rate of each ammonia species is proportional to its environmental concentration and if the

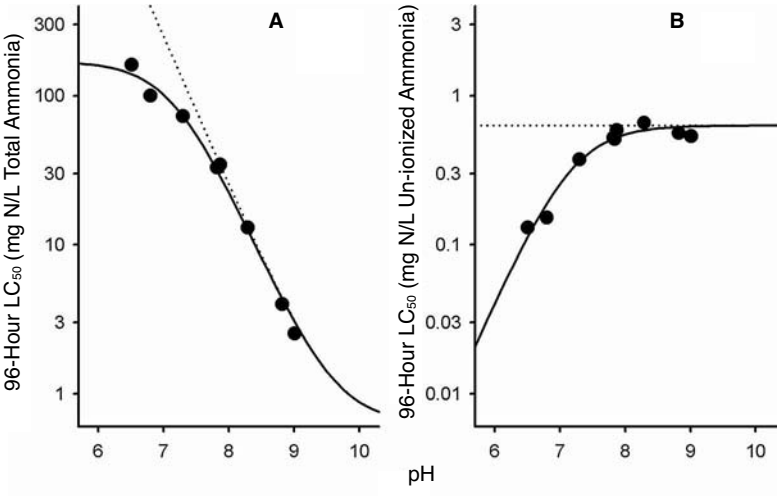

FIGURE 2.5 Effects of pH on toxicity of ammonia to rainbow trout expressed as a function of (A) total ammonia and (B) un-ionized ammonia. The solid lines denote fit of data to a model in which both un-ionized ammonia and ammonium ion contribute to bioavailability. The dotted lines denote expected effects of pH if only un-ionized ammonia is bioavailable. (Data from Thurston, R.V. et al., *Environ. Sci. Technol.*, 15, 837–840, 1981.)

elimination rate of ammonia is proportional to its total concentration within the fish, the accumulation of ammonia at a site of interest within a fish (C_F) can be described by the differential equation:

$$\begin{aligned}\frac{dC_F(t)}{dt} &= k_{U,NH_3}\cdot C_{NH_3}(t) + k_{U,NH_4^+}\cdot C_{NH_4^+}(t) - k_R\cdot C_F(t)\\ &= \left(k_{U,NH_3}\cdot f_{NH_3} + k_{U,NH_4^+}\cdot f_{NH_4^+}\right)\cdot C_{TAMM}(t) - k_R\cdot C_F(t)\end{aligned} \tag{2.2}$$

where C_{TAMM}, C_{NH_3}, and $C_{NH_4^+}$ are the environmental concentrations of total ammonia, un-ionized ammonia, and ammonium ion, respectively; f_{NH_3} and $f_{NH_4^+}$ are the fractions of the total ammonia consisting of un-ionized ammonia and ammonium ion, respectively; k_{U,NH_3} and k_{U,NH_4^+} are uptake rate constants for the un-ionized ammonia and ammonium ion species; and k_R is the elimination rate constant. When the exposure concentration is constant, this differential equation can be integrated to provide the following algebraic expression for C_F:

$$C_F(t) = \frac{k_{U,NH_3}\cdot f_{NH_3} + k_{U,NH_4^+}\cdot f_{NH_4^+}}{k_R}\cdot\left(1-e^{-k_R t}\right)\cdot C_{TAMM} \tag{2.3}$$

If a given level of effect is associated with a certain level of ammonia accumulation (EC_F), then the total ammonia environmental concentration required to elicit this effect (EC_{TAMM}) for an exposure duration t_E can be derived by substituting EC_F for C_F and EC_{TAMM} for C_{TAMM} in the above equation and rearranging as follows:

$$EC_{TAMM} = \frac{1}{\dfrac{f_{NH_3}}{EC_{NH_3}} + \dfrac{f_{NH_4^+}}{EC_{NH_4^+}}}, \quad \text{where } EC_x = \frac{k_R\cdot EC_F}{k_{U,x}\left(1-e^{-k_R t_E}\right)} \tag{2.4}$$

where EC_{NH_3} is the effect concentration when only un-ionized ammonia is present, and $EC_{NH_4^+}$ is the effect concentration when only ammonium ion is present. EC_{TAMM} is therefore a weighted harmonic mean of EC_{NH_3} and $EC_{NH_4^+}$, the weighting factors being the fractional concentrations of the species in the exposure water. Substituting formulas for f_{NH_3} and $f_{NH_4^+}$ as a function of pH results in the following:

$$EC_{TAMM} = \frac{1 + 10^{pK_a - pH}}{\dfrac{1}{EC_{NH_3}} + \dfrac{10^{pK_a - pH}}{EC_{NH_4^+}}} \tag{2.5}$$

This describes a sigmoidal relationship of log EC_{TAMM} to pH, with the value for EC_{TAMM} approaching EC_{NH_3} at high pH and $EC_{NH_4^+}$ at low pH.

Erickson (1985) reported a good fit of the above model to the data in Figure 2.5A, with $LC_{50\,NH_3}$ estimated to be 0.63 mg N per liter and $LC_{50\,NH_4^+}$ estimated to be 170 mg N per liter. Substituting these values and $pK_a = 9.6$ (the value for 13.5°C, the average temperature in the study) into Equation 2.5 produces the solid line in Figure 2.5A, and multiplying these values by f_{NH_3} produces the solid line in Figure 2.5B. The good fit of this model depends not only on these estimated values for $LC_{50\,NH_3}$ and $LC_{50\,NH_4^+}$ but also on the adherence of the data to the model assumption that $LC_{50\,TAMM}$ is a weighted average of $LC_{50\,NH_3}$ and $LC_{50\,NH_4^+}$, with the weighting factors being the fractional species concentrations. An important consequence of this assumption is that $LC_{50\,TAMM}$ closely parallels the fractional concentration of the more bioavailable form (dotted lines in Figure 2.5) until this fraction is low enough that the less bioavailable form is responsible for a significant portion of the toxicity.

Other datasets on ammonia toxicity to fish also indicate that pH effects can be primarily attributed to joint toxicity of un-ionized ammonia and ammonium ion (Erickson, 1985); however, the relative bioavailabilities of these two chemical species can differ among fish species. The ratio of $LC_{50\,NH_3}$ to $LC_{50\,NH_4^+}$ varied from 0.001 to 0.006, mainly attributable to differences in the bioavailability of ammonium ion for different fish. In particular, studies with channel catfish (Sheehan and Lewis, 1986; Tomasso et al., 1980) have suggested little or no bioavailability of ammonium ion, with nearly constant LC_{50} values on the basis of un-ionized ammonia. Such differences among fish species may be related to differences in ionoregulatory and ammonia excretion mechanisms, but this has not been investigated.

Ammonia bioavailability to fish, however, is not just a matter of the above simple model. Several additional factors that can make ammonia bioavailability relationships more complex may have to be considered in specific assessment situations:

1. Excretion of respiratory carbon dioxide will depress the pH at the gill surface. This will cause a shift in equilibrium of ammonia speciation, resulting in less un-ionized ammonia and more ammonium ion at the gill surface than in the bulk exposure water. The degree of this effect depends on the exposure pH, the pH-buffering strength of the exposure water, and the physiology of the fish. Lloyd and Herbert (1962) and Szumski et al. (1982) suggested that this, rather than some bioavailability of ammonium ion, could account for toxicity on a un-ionized ammonia basis not being constant with pH. The analyses of Erickson (1985), however, indicated that this mechanism is generally of secondary importance to the joint toxicity of un-ionized and ammonium ion.
2. Ammonia is unique among toxicants of concern for fish because it is also an excretory product, and this endogenous ammonia will contribute to some degree to the accumulation of ammonia to toxic levels. If excretion rates are not constant with pH, this can affect the pH dependence of the relationship of toxicity to exogenous ammonia. High pH can inhibit ammonia excretion because more of the released ammonia remains in the un-ionized form, which reduces the blood–water gradient of un-ionized ammonia and thus the net diffusive loss. Russo et al. (1988) reported increased ammonia toxicity at pH values near and above 9 which might be related to such effects. Exogenous ammonia might also affect excretory mechanisms, thereby altering ammonia accumulation other than by direct uptake.
3. Sheehan and Lewis (1986) suggested that acute ammonia toxicity to channel catfish at low pH is partly attributable to osmotic effects of high ammonium salt concentrations (>1000 mg N per liter). To the extent their conclusions are true, the ammonium ion might be essentially nonbioavailable and exert its effects simply based on its contribution to the external osmotic pressure. Although this might be important for channel catfish, other fish species show apparent effects of ammonium ion at much lower concentrations (ca. 100 mg N per liter) where such

osmotic effects are unlikely to be as significant. Nonetheless, this is a possibility that should be recognized in more comprehensive evaluations of ammonia bioavailability.

4. The bioavailability of ammonia might also be affected by other ionic constituents of the exposure water. Ammonia toxicity to fish has been reported to be reduced due to increases in the hardness or salinity of test water. Causes for this have been suggested in work with the amphipod *Hyalella azteca*. Ankley et al. (1995) reported ammonia toxicity to *H. azteca* to be lower in hard test water than in soft test water, especially at low pH. Borgmann and Borgmann (1997) further found that the toxicity of ammonia to *H. azteca* was reduced by sodium, but not by calcium, and suggested that this effect is greater at low pH because mechanisms for transport of ammonium ion across fish gills are affected by the ionic composition of the water, whereas the passive transport of un-ionized ammonia is not.
5. Because the fraction of the more bioavailable un-ionized ammonia increases with temperature, it might be expected that total ammonia toxicity would increase with temperature; however, for fish, there is little effect of temperature on ammonia toxicity expressed on the basis of total ammonia (U.S. EPA, 1999). Erickson (1985) noted that the effect of temperature on ammonia toxicity is inconsistent with the relative toxicities of un-ionized ammonia and ammonium ion inferred from the pH dependence of ammonia toxicity. The effects of temperature on ammonia toxicity thus apparently reflect multiple influences on ammonia metabolism, excretion, and toxicological response that are not yet well understood.

Evaluations of ammonia bioavailability, therefore, can involve various considerations beyond the simple model presented above, depending on the exposure circumstances, the fish species of concern, and the level of detail and precision desired in an assessment. Nonetheless, the pH dependence of ammonia toxicity still provides a good example of how the bioavailability of a chemical, to a good approximation, can reflect a simple weighted average of contributions of its constituent species. This type of model has broad applicability to bioavailability assessments.

Ionizable Organic: Phenol Derivatives

Some organic chemicals contain functional groups that are weak acids or bases. Such chemicals will exist as multiple species with different charges at these functional groups, which can affect how readily they will be accumulated by a fish. The relative amounts of these chemical species will vary with pH, so the bioavailability of the chemical can also vary with pH.

A group of chemicals that has received considerable study is phenol and its various substituted derivatives, especially pentachlorophenol (PCP), which has been extensively used as a wood preservative and is a widespread contaminant of soils and aquatic systems. This class of chemicals consists of a hydroxyl group (OH) on a phenyl ring (Φ) and can exist as un-ionized molecules (RΦOH) or as phenolate ions ($R\Phi O^-$), where R denotes other functional groups on the ring. These two chemical species are in equilibrium with each other according to the following expression:

$$K_a = \frac{[H^+][R\Phi O^-]}{[R\Phi OH]} \tag{2.6}$$

where the acid dissociation constant (K_a) varies with the nature of R. In the subsequent discussion, the term *phenol* refers to any member of this class of chemicals.

When based on concentrations in water, the toxicity and accumulation of various phenols in fish have been found to decrease with increasing pH (see Figure 2.6). The inverse relationship between uptake rate constants and toxic water concentrations in Figure 2.6 suggests that the degree of accumulation required to elicit a given level of toxicity does not vary much with pH. This is more evident in Figure 2.7, which shows that LC_{50} values for PCP increase with pH by about the same factor as the BCFs decrease, resulting in estimated body burdens at death being approximately constant with pH. Kobayashi and Kishino (1980) reported that PCP concentrations measured in dead fish were similar at different

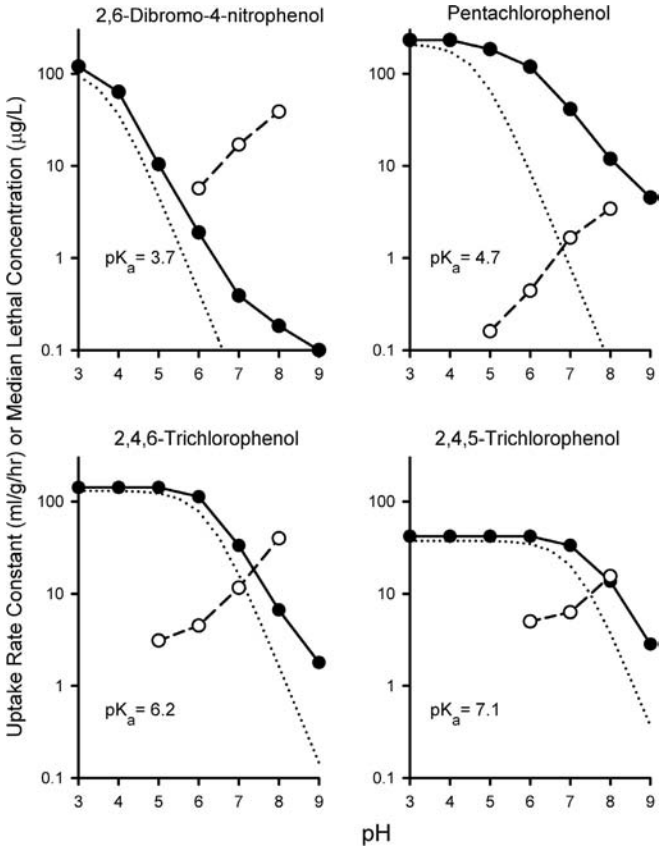

FIGURE 2.6 Observed pH dependence of uptake rate constants (closed circles; see Saarikoski et al., 1986) and median lethal concentration (open circles; see Saarikoski and Viluksela, 1981) of several substituted phenols for guppies. Dotted line denotes the expected uptake rate constant based on the value of the constant at pH 3 and the assumption that uptake is proportional to the fraction of the chemical in exposure water that is un-ionized.

pHs. This indicates that the pH dependence of toxic water concentrations is largely attributable to processes that affect accumulation; thus, toxicity as well as accumulation can be an indicator of bioavailability, at least for assessing the effects of pH.

Because the relative amount of un-ionized phenol decreases and that of phenolate ion increases with increasing pH, a likely explanation for these effects of pH on phenol bioavailability is that the ionic form is less readily accumulated. This is a reasonable expectation because fish gill epithelial cell membranes should be more permeable to un-ionized phenol molecules than to their phenolate ions. This explanation is further supported by the observation that significant effects of pH are only observed when the pH is near or above the pK_a of a particular phenol (Figure 2.6). For pH below the pK_a, the phenol is almost entirely in the un-ionized form, so phenol bioavailability should not depend greatly on environmental pH. At higher pH, the relative amount of the un-ionized species declines appreciably with increasing pH, thereby reducing the overall bioavailability of the mixture of species.

The effect of pH on bioavailability, however, is not simply a matter of the un-ionized phenol in the exposure water being the sole basis for accumulation. If this were the case, the observed uptake rate constants should decline in proportion to the fraction of the phenol that is not ionized (dotted lines in Figure 2.6). Instead, once a chemical is appreciably ionized, uptake is greater than expected based on the un-ionized phenol in the exposure water, sometimes by more than an order of magnitude. For uptake of several chlorinated phenols by large rainbow trout, Erickson et al. (2006b) reported even greater deviations of uptake rate constants from that expected based on the amount of un-ionized phenol (see Figure 2.8).

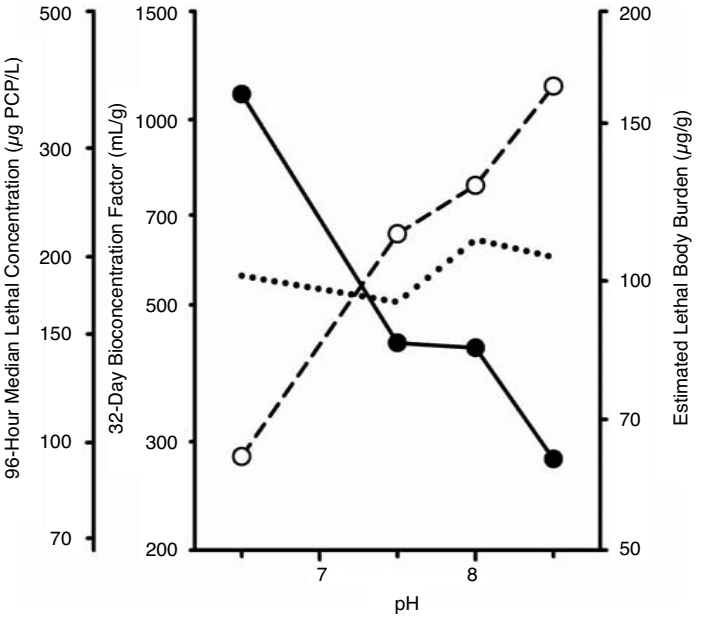

FIGURE 2.7 Observed pH dependence of median lethal concentrations (open circles) and bioconcentration factors (closed circles) of pentachlorophenol for juvenile fathead minnows. Dotted line denotes estimated lethal body burden as product of bioconcentration factor and median lethal concentration. (Data from Spehar, R.L. et al., *Environ. Toxicol. Chem.*, 4, 389–397, 1985.)

Such high uptake rates in the presence of substantial ionization indicate that phenolate ions do contribute in some way to phenol bioavailability. The processes within fish gills discussed earlier (Figure 2.3 and associated text) are responsible for this, and three specific mechanisms have been suggested to be important for phenol bioavailability (Erickson et al., 2006a; McKim and Erickson, 1991; Saarikoski et al., 1986).

The first mechanism arises from rapid kinetics for the interconversion of the un-ionized phenol and phenolate ion. When un-ionized phenol diffuses across the outer gill epithelium cell membrane, its concentration in the water adjacent to the gill surface is depleted, but this depletion will be moderated by a rapid net conversion of the phenolate ions to the un-ionized species to maintain the chemical equilibrium represented by Equation 2.6. The phenolate ion thus acts as a buffer, maintaining the concentration of the uncharged form adjacent to the gill surface higher than it would be in the absence of the ion or if the kinetics of this reaction were slow. In addition, efficient uptake depends on rapid diffusion of chemical across the gill lamellar water channels to the epithelial surface. The presence of phenolate ion will facilitate uptake by contributing to this diffusion.

The importance of these rapid speciation changes does not stop at the external gill surface. After crossing the outer cellular membrane, there will be net conversion of un-ionized phenol molecules to phenolate ions to maintain equilibrium within the cytosol of the epithelial cells. This increases the diffusive gradient, and thus the rate of diffusion, across this membrane. In addition, the phenolate ions so formed will contribute to diffusion across the cytosol, increasing diffusive transport compared to what would occur if slow kinetics kept all the absorbed chemical in the un-ionized form. This process will be repeated at each membrane and any other diffusion barriers that are more permeable to the un-ionized molecules than to phenolate ions. The phenolate ions thus facilitate diffusion across the epithelium by supporting diffusion to and from the membranes and by helping to maintain steeper gradients of the un-ionized phenols across the membranes than would otherwise exist. Because membranes constitute a small fraction of the total diffusion path across the epithelium, this mechanism can result in gradients across the membranes steep enough to maintain high rates of diffusion even if little of the phenol is in its un-ionized form.

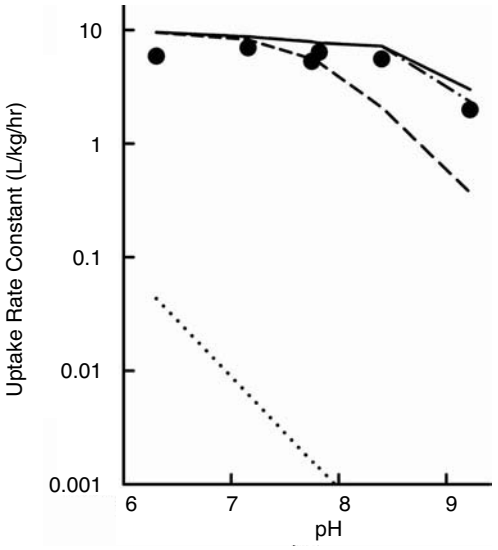

FIGURE 2.8 Observed (closed circles) and predicted (solid line) rate constants vs. pH for PCP uptake by large rainbow trout. Dashed–dotted line denotes predictions when the model was modified to have no membrane permeability of pentachlorophenolate ion. Dashed line denotes predictions when the model was also modified to have no pH changes in water passing through the gill. Dotted line denotes predictions when the model was also modified to have no interconversion of pentachlorophenolate ion and un-ionized pentachlorophenol. (Data from Erickson, R.J. et al., *Environ. Toxicol. Chem.*, 25, 1522–1532, 2006.)

A noteworthy feature of this mechanism is that chemical uptake depends not just on ionization in the exposure water but also on ionization within the organism. This might explain the higher uptake rate constant at low pH for 2,4,6-trichlorophenol than for 2,4,5-trichlorophenol in Figure 2.6. In the exposure water, both chemicals are >99% in their un-ionized forms at the lowest pH and should be similar in their diffusive properties and partitioning relationships; however, both chemicals will be appreciably ionized at the higher pH within fish tissue. Because the 2,4,6-trichlorophenol has a lower pK_a, it will be more ionized than 2,4,5-trichlorophenol within the epithelial cytosol and in the blood, which could account for the difference in their uptake rates.

The second mechanism by which phenolate ion can contribute to phenol bioavailability arises from fish respiration causing a reduction of the pH in the water passing through gill lamellar channels (Lloyd and Herbert, 1960; Playle and Wood, 1989; Wright et al., 1991). This reduction in pH will increase the fraction of the more bioavailable un-ionized phenol, increasing uptake beyond that expected based on the concentration of un-ionized phenol in the bulk exposure water. This mechanism would shift the uptake rate constant vs. pH curve to a higher pH, the degree of this shift being roughly equal to the difference between the pH of the bulk exposure water and the average pH at the gill surface. The magnitude of the increased uptake will depend on the magnitude of the pH reduction at the gill surface and on how sensitive uptake is to changes in pH at the gill surface, which in turn depend on the pH and alkalinity of the exposure water, the morphology and physiology of the fish, and the pK_a and other properties of the chemical. Erickson et al. (2006b) demonstrated that increased alkalinity reduces phenol uptake by moderating the pH reduction in water passing through the gills.

For these first two mechanisms, the effects of phenolate ion on bioavailability depend on its interconversion with un-ionized phenol and require diffusion barriers that are more permeable to the un-ionized than the ionized species. The third mechanism of interest here is that gill epithelia do not contain continuous diffusion barriers completely impermeable to phenolate ions. Saarikoski et al. (1986) suggested the possibility of phenolate ions bypassing membranes by passing through gill epithelial cell junctions; however, some partitioning of phenolate ions into organic solvents and lipid membranes and some mobility of phenolate ions within membranes have been reported (Escher and Schwarzenbach, 1996; Escher et al., 1999; Jafvert et al., 1990; Smejtek et al., 1996), suggesting the possibility of some phenolate diffusion across membranes. Whatever the mechanistic details, this would result in sigmoidal

bioavailability relationships similar to that discussed above for ammonia, with the lower part of this sigmoidal curve reflecting the contribution of phenolate ions. This mechanism would explain reduced slopes of uptake vs. pH when uptake becomes low at high pH, such as can be observed for three of the chemicals in Figure 2.6; however, it could not explain high rates of uptake in the presence of substantial ionization.

Figure 2.8 explores the relative importance of these mechanisms by applying a mathematical model for predicting gill uptake of ionizable organic chemicals (Erickson et al., 2006a,b). In the absence of the above mechanisms by which phenolate ion can contribute to uptake, the model-predicted uptake (dotted line) is extremely low because PCP is so highly ionized, and is two to four orders of magnitude below the observed uptake rate constants. This rate would be expected if the epithelium contained barriers completely impermeable to the phenolate ion and if the kinetics of interconversion between the un-ionized and ionized species were slow compared to transport processes in the gill. Adding the first mechanism discussed above (rapid interconversion of phenolate ion and un-ionized phenol) to the model (dashed line) accounts for most of the difference between the dotted line and the data. Also, adding pH changes in gill water to the model (dash-dotted line) provides additional improvement to model predictions at high pH, bringing the predictions very close to the observed data. Adding the final mechanism (some permeability of cellular membranes to the phenolate ion) to the model has only a small effect on the full model predictions (solid line), because the rate constants in this dataset are relatively high.

The example in Figure 2.8 should not be treated as being generally reflective of the relative importance of these different mechanisms to phenol bioavailability. As already noted, membrane permeability for the phenolate ion will become more important when uptake rates drop to levels lower than addressed in this example, such as in Figure 2.6. The rapid interconversion of phenolate ion and un-ionized phenol has a particularly large effect in the example in Figure 2.8 but appears to be less important for the conditions of PCP uptake in Figure 2.6 and even less important for the other chemicals in Figure 2.6. The relative importance of these mechanisms will vary substantially depending on properties of the chemical, fish, and exposure of interest.

This section considered a more complicated bioavailability situation than for ammonia, addressing a variety of the physical, chemical, and biological processes introduced earlier in Figure 2.3. Of particular note is that a chemical species in exposure water can contribute to bioavailability even if it is not directly absorbed by the organism, because of changes in chemical speciation that occur within the gill. Phenol bioavailability might be even more complicated due to processes and factors not discussed here (e.g., the influence of other chemical constituents, such as organic matter, on phenol speciation), so the material here should be treated as an example of how to approach certain aspects of bioavailability assessments, not as a comprehensive discussion of all aspects of phenol bioavailability.

The mechanisms discussed above for the contribution of phenolate ion to phenol uptake can also affect phenol elimination (Erickson et al., 2006b), and this can have consequences for bioavailability assessments. Because any such effects on elimination are more important for steady-state accumulation than during the initial stages of accumulation, the pH dependence of phenol accumulation would be expected to vary with time. Available data do not allow confirmation of such an effect of time on bioavailability, but this possibility illustrates how the meaning of bioavailability might be conditional on the specific exposure situation and must be carefully defined.

Cationic Metals: Copper

The assessment of the risks of metals to fish is often difficult because of large and complex effects of exposure water characteristics on metal toxicity. The nature and magnitude of these effects can differ among metals, toxicity endpoints, fish species, and routes of exposure. This section focuses on the acute lethality of copper to freshwater fish which has been reported by various authors to vary with pH, hardness, alkalinity, suspended solids, dissolved organic compounds, and various other inorganic cations and anions. Most, although not all, of this variation in toxicity can be attributed to effects of these physicochemical factors on copper bioavailability.

Some understanding of toxicity mechanisms can help in understanding the effects of environmental factors on toxicity and bioavailability. Acute copper lethality in freshwater fish has been related to disruption of osmoregulation at fish gills. In particular, copper has been demonstrated to reduce sodium

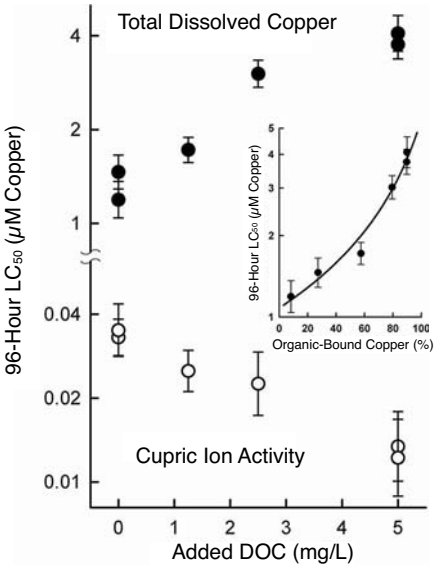

FIGURE 2.9 Effects of humic acid additions on acute copper toxicity to fathead minnows. The main figure shows observed LC_{50} values on the basis of both dissolved copper concentrations and cupric ion activities vs. the amount of added dissolved organic carbon (DOC). The inset figure shows dissolved copper LC_{50} values vs. the fraction of copper bound by the organic matter, with the symbols denoting the observed LC_{50} values and the line denoting expected trends if the organic-bound copper is 20% as bioavailable as copper not bound to organic carbon. (Data from Erickson, R.J. et al., *Environ. Toxicol. Chem.*, 15, 181–193, 1996.)

uptake and increase sodium loss at fish gills, with death occurring because of decreased blood sodium concentration (Lauren and McDonald, 1986; Paquin et al., 2002a,b; Wood, 1992; Wood et al., 1997). An important component of sodium uptake that can be affected by copper is the active transport of sodium via Na^+/K^+-ATPase across basal membranes of gill epithelial cells. Copper toxicity therefore is related to how readily copper is taken up at the apical (external) membrane of gill epithelial cells to reach this site of action. Sodium uptake across these apical membranes is coupled to the release of hydrogen ion, and these processes might also be important in the relationship of copper toxicity to environmental factors. Sodium loss at gills is largely via passive diffusion through paracellular junctions, and copper toxicity increases this loss. Calcium plays an important role in regulating the permeability of these junctions and might thereby also affect copper toxicity.

Many of the factors affecting copper toxicity cited above also affect copper speciation and thereby copper bioavailability. Copper in oxygenated water will be in a positive oxidation state, predominantly Cu(II), which will be present to some degree as a hydrated cation, $Cu^{+2}{\cdot}nH_2O$, often referred to as *free copper ion*. Free copper has a high affinity for various ligands found in natural waters, including hydroxide, carbonate, sulfide, and various dissolved organic molecules; consequently, dissolved copper in freshwater mostly exists as complexes with such ligands. Copper also adsorbs to mineral and organic particles, and in saline water copper is largely complexed by chloride. These various species generally are in rapid flux, with any specific copper atom associating with, and dissociating from, various ligands. The fraction of copper that exists as a particular species is not some fixed subset of the copper atoms but rather the portion of the copper atoms existing as that species at a given moment. As discussed above for phenol, this dynamic speciation can have important consequences for bioavailability. Kramer et al. (1997), Bryan et al. (2002), and Smith et al. (2002) provide useful starting points for further information on copper speciation.

Increased complexation of copper by various inorganic and organic ligands generally is associated with decreased toxicity, suggesting that these complexes are less bioavailable than the free metal (see reviews by Campbell, 1995; Hunt, 1987; Paquin et al., 2002a; Sprague, 1985). Figure 2.9 provides an example of this, where the addition of humic acid (5 mg C per liter) increased the median lethal concentration of total dissolved copper to fathead minnows by more than threefold. The inset on Figure 2.9 indicates that this

decrease in toxicity was associated with an increase in the fraction of copper that was complexed by organic matter. That these effects on toxicity reflect changes in bioavailability is supported by studies that have demonstrated that accumulation of copper in fish gills and other tissues is reduced by complexation of copper with organic compounds in exposure water (Buckley et al., 1984; MacRae et al., 1999; Muramoto, 1980; Playle et al., 1993a,b).

Some studies, especially with phytoplankton and zooplankton, have shown a given level of toxicity to be associated with a constant activity of free copper over a wide range of total copper, suggesting that some copper complexes contribute little, if anything, to bioavailability (see review by Campbell, 1995). In other cases, however, toxicity on the basis of free copper activity has been reported to increase as complexation by various inorganic and organic ligands increases, suggesting some contribution of copper complexes to bioavailability (see reviews by Campbell, 1995; Hunt, 1987; Paquin et al., 2002a; Sprague, 1985). This bioavailability might arise from release of free copper from a complex in the microenvironment at the gill surface or by a copper complex directly crossing over, or interacting with, the gill epithelial surface. In some cases, organic ligands can even increase copper toxicity, presumably because certain hydrophobic complexes of copper are able to more readily cross lipid cellular membranes (Florence and Stauber, 1986). The study summarized in Figure 2.9 suggested some bioavailability of the organic-complexed copper, because, although the addition of humic acid reduced the toxicity of copper to fathead minnows on the basis of total dissolved concentrations, toxicity on the basis of free copper ion was increased. The line in the inset in Figure 2.9 indicates the fit of a joint toxicity model (such as that used for ammonia above), for which the humic-complexed copper is estimated to be about 20% as toxic as the rest of the copper. Such a contribution to bioavailability by copper complexed to such large ligands could be due to partial dissociation of these complexes at the gill surface, perhaps because of the reduced pH expected at this surface; however, in the absence of direct evidence from accumulation measurements, other explanations for the apparent bioavailability of organic-complexed copper cannot be completely discounted. The organic matter might also affect the speciation of other ionic constituents important in copper toxicity or exert some direct effect on the gill surface.

Although copper speciation and its role in bioavailability can explain the effects on toxicity of many of the physicochemical factors cited at the start of the section, some of these factors will have little or no effect on copper speciation in the exposure water. One such factor is water hardness, which refers to certain cationic components in the water, primarily calcium and magnesium. Such cations would not affect aqueous copper speciation except to the extent that they compete with copper for ligands, and such effects are generally so small that they could not be responsible for any appreciable effects on toxicity. Increased hardness has been long reported to be associated with decreased metal toxicity, but the nature of these effects is not particularly clear or consistent. In some cases, reported effects of hardness are actually combined effects of hardness, pH, and alkalinity, so any effects of the hardness cations were confounded with those of copper complexation by hydroxide and carbonate. In several studies with fish, however, hardness was varied in association with anions that do not significantly affect copper speciation. Usually, reductions in toxicity were still found (Chakoumakos et al., 1979; Erickson et al., 1987; Inglis and Davis, 1972; Miller and Mackay, 1980), an example of which is shown in Figure 2.10. In other cases, little or no effect of hardness was found (Lauren and McDonald, 1986; Zitko and Carson, 1976). The effect of hardness can also depend on the relative amounts of calcium and magnesium and on the test species (Naddy et al., 2002). Figure 2.10 suggests that the incremental effects of hardness are greater at low hardness than at high hardness. This nonlinearity is further evident in other data for fathead minnows from this same study (Erickson et al., 1987) and from studies with cladocerans (Gensemer et al., 2002). To further complicate the situation, Lauren and Macdonald (1986) proposed that apparent effects of hardness on acute copper toxicity can be affected by inadequate acclimation of fish to the hardness, and that hardness effects could be manifested more strongly in longer exposures. Erickson et al. (1997), however, found similar effects of hardness on acute toxicity for both acclimated and unacclimated fish.

Zitko (1976), Zitko and Carson (1976), and Pagenkopf (1983) suggested that hardness cations can affect metal toxicity by competing for biochemical receptors on fish gills involved in the uptake or effects of toxic metals, thereby necessitating higher concentrations of the toxic metal to result in enough toxic metal accumulation on these receptors to elicit effects. The uptake of copper might also be affected by the effects of calcium on the permeability of paracellular junctions. Such mechanisms would represent

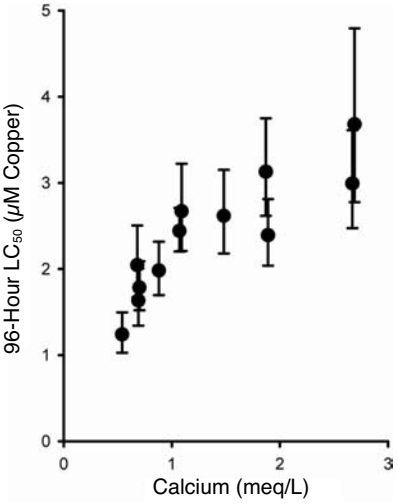

FIGURE 2.10 Effects of calcium on acute copper toxicity to fathead minnows. Symbols denote observed LC_{50} values (±95% confidence limits) on the basis of dissolved copper. Just the calcium component of hardness is included because it was considered to be the primary source of hardness effects in this study. (Data from Erickson et al., *A Prototype Toxicity Factors Model for Site-Specific Copper Water Quality Criteria*, U.S. Environmental Protection Agency, Duluth, MN, 1987.)

effects on copper bioavailability because they concern how much metal is taken up relative to the total amount of metal in the exposure water; however, the effects of calcium on gill permeability might also simply make it more or less difficult for a fish to osmoregulate and thus change its susceptibility to copper without altering copper bioavailability.

Little copper accumulation information is available to demonstrate to what degree hardness effects on copper toxicity represent actual changes in copper bioavailability. Playle et al. (1992) demonstrated that copper accumulation in fish gill tissue is reduced by increased calcium concentrations under some exposure conditions, but they also found no such effects for other conditions under which effects of hardness on toxicity might be expected. In contrast, good demonstrations that hardness affects toxicity by reducing metal accumulation have been provided by Meyer et al. (2002) for copper toxicity to oligochaete worms and by Meyer et al. (1999) for nickel toxicity to fathead minnows.

Cations other than those associated with water hardness might also influence copper bioavailability. Erickson et al. (1987) reported that increased sodium concentrations reduced toxicity. Because copper toxicity involves disruption of sodium exchange, this ameliorative effect of sodium might not be an issue of bioavailability but rather might simply reflect a more favorable gradient for sodium uptake, which would necessitate more copper accumulation to disrupt osmoregulation enough to elicit toxicity. For silver, however, Janes and Playle (1995) reported that increased sodium reduced metal accumulation in gills. Whether sodium has similar effects on copper bioavailability is uncertain. Hydrogen ion also could be a competitor with metals for gill binding sites and a modifier of gill epithelial properties; thus, pH could influence copper bioavailability beyond its effects on copper speciation in the exposure water. The effects of pH on toxicity could therefore be quite complex, with several processes altering bioavailability as pH changes. Although not clearly demonstrated in fish, competitive effects of hydrogen ion have been strongly indicated in some algal copper toxicity data (Peterson et al., 1984).

The various effects of water chemistry on copper bioavailability discussed thus far are summarized in the conceptual framework shown in Figure 2.11. For simplicity, Figure 2.11 depicts just two species of copper: free copper ion and copper complexed by a ligand (L), which has an unspecified charge and represents the various constituents in the exposure water or within the fish that can complex copper. The vertical arrows connecting Cu^{+2} and CuL represent the association and dissociation reactions that are continually occurring among the various forms. Free and bound copper in the bulk exposure water are transported by advection and diffusion (horizontal arrows) to near the gill surface, into a chemical microenvironment created by the gill. These arrows converge to indicate that all species contribute to

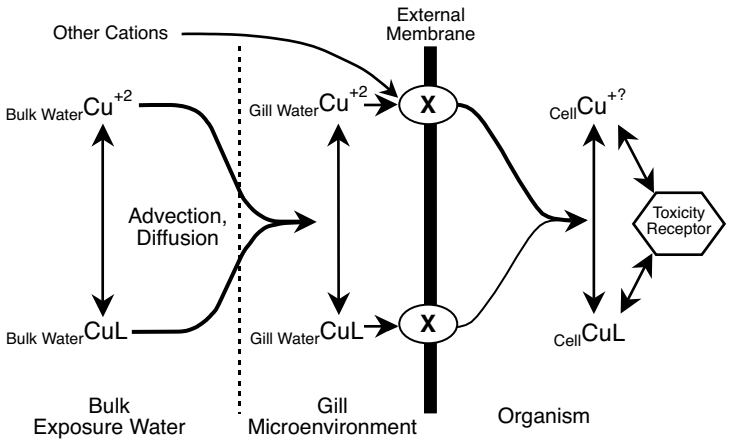

FIGURE 2.11 Conceptual model for chemical interactions regulating copper bioavailability at fish gills. See text for explanation of symbols.

the total pool of copper within the gill microenvironment. The various speciation reactions will result in a distribution of copper species within the gill microenvironment different from that in the bulk exposure water. These reactions are not necessarily at equilibrium, so bioavailability also can be a function of the kinetics of these reactions.

Some of these species will interact with or cross the external surface of the gill by various mechanisms denoted by the ovals marked with an X in Figure 2.11. The extent to which copper is absorbed by the fish and reaches internal toxicity receptors is represented by the horizontal arrows penetrating the surface. These arrows are of different thicknesses to denote that the gill surface is often less permeable, but not necessarily completely impermeable, to copper bound to ligands. Other cations, such as calcium, hydrogen, and sodium, might exert an effect on copper bioavailability by competing with copper for sites on the gill surface, or otherwise affecting the permeability of the gill surface to copper. The arrows again converge on the other side of the membrane to indicate that any copper entering the organism contributes to the total pool of copper within cells. This pool of copper is further affected by various speciation reactions (including changes in copper oxidation state) and is transported to where the different chemical species can interact with toxicity receptors.

Many of the processes and concepts depicted in Figure 2.11 have been the basis for metal bioavailability models proposed by various authors. These include model equations presented by Zitko (1976), the *gill surface interaction model* described and evaluated by Pagenkopf (1983), the *free ion activity model* (FIAM) described by Morel (1983), further developments of the FIAM by Brown and Markich (2000), and more recently the *biotic ligand model*, so named because biochemical sites such as those marked with an X in Figure 2.11 are viewed as other ligands that the metal interacts with (DiToro et al., 2001; Meyer et al., 1999; Santore et al., 2001). Reviews and critiques of these models are available from Campbell (1995) and Paquin et al. (2002a).

These models have similar basic features. Toxicity is a function of the amount of metal bound to a site on the organism surface. This is not necessarily a site of toxic action but rather might be an uptake site that defines how much metal gets to the toxicity receptors. This site is of central importance to bioavailability because its external location allows interaction with the exposure water chemistry. The metal bound at this site is assumed to be in equilibrium with the free metal in the water to which it is exposed. Complexation by various ligands in the exposure water will therefore limit the amount of metal binding to the site. Various cations can also bind with the site, creating competitive interactions that also limit binding of the toxic metal. The models include, or can be readily modified to include, binding of other species of the toxic metal at the site as mixed ligand complexes, so these species can also contribute to uptake and toxicity. Of course, it is an oversimplification to depict all relevant effects as arising from simple chemical mass action of these chemical entities at a single type of site; however, such a mathematical treatment can still be a useful approximation for the processes that do occur.

When just the free form of the toxic metal (M) binds to the gill sites (X), this model can be expressed as follows:

$$EC = \left(\frac{f_{EX}}{(1 - f_{EX}) \cdot K_{MX}} \right) \cdot \left(1 + \sum_{j}^{m} \left(K_{CjX} \cdot [C_j] \right) \right) \cdot \left(1 + \sum_{i}^{n} R_i \right) \quad (2.7)$$

where EC is the total concentration of metal in the exposure water required to elicit a specified effect and is a product of the three mathematical expressions on the right side of the equation. The first expression is a baseline effect concentration that is expected in the absence of any cation competition and ligand complexation. It is a function of (1) f_{EX}, the fraction ($[MX_{EFF}]/[X_{TOT}]$) of the gill sites that must be occupied by the toxic metal to elicit the effect of concern, and (2) K_{MX}, the association constant for the binding of the toxic metal to the gill sites.

The second expression provides the factor by which the EC increases (bioavailability decreases) due to cations that compete with the toxic metal for the gill sites. This factor increases with increasing products of $[C_j]$, the free concentration of the j-th of m competing cations, and K_{CjG}, the association constant for the binding of this cation to the gill sites.

The third expression provides the factor by which the EC increases (bioavailability decreases) due to ligands that complex the toxic metal in the exposure water. This factor increases with increasing concentration ratios (R_i) of each metal complex to the free metal. For single-ligand complexes, this ratio is simply the product of the free concentration of the ligand and the association constant for this complex; however, in general, calculation of the R_i values requires a chemical speciation model that can address more complicated speciation relationships and relate calculations to total chemical concentrations, because free concentrations generally are unknown.

When only one competing cation or complexing ligand is varied, this model predicts a linear relationship between the EC and the free concentration of that cation or ligand, with the intercept equal to the baseline EC. De Schamphelaere and Janssen (2002) provided good examples of such linear relationships for copper toxicity to a cladoceran and also addressed how this mathematical framework can be expanded to address the toxicity of species other than free metal.

Recent efforts have demonstrated that this bioavailability modeling approach can be useful for describing the toxicity of various metals to fish and other aquatic organisms (see review by Paquin et al., 2002a). Santore et al. (2001) and Di Toro et al. (2001) accounted for a high percentage of the variation of toxicity within a large dataset for acute copper lethality to fathead minnows, nearly always predicting toxicity to within a factor of two. Figure 2.12 shows a subset of these data consisting of a series of test waters with the same pH, low organic matter content, and similar relative amounts of major cations and anions but different total ion concentrations. The large range of copper toxicity in this dataset primarily reflects the combined effects of changes in alkalinity, calcium, and sodium. The model is very successful in predicting relative changes in toxicity and underestimates LC_{50} values by only about 30% on average. This underestimation of LC_{50} values might be due to uncertainties in model parameterization and sample characterization (especially regarding the organic complexation in these samples) or to uncertainties in model formulation discussed below.

One strength of this modeling approach is that parameters can be primarily, or even entirely, estimated independently of the toxicity relationships of interest. Estimation of metal speciation in exposure water does not depend at all on toxicity data, and association constants for binding of metal to gill sites can be estimated from accumulation experiments. Only the gill accumulation associated with the toxic effect has to be determined under toxic conditions, and it does not have to involve any consideration of the effects of exposure conditions on toxicity (MacRae et al., 1999). If such independent parameter estimates are not feasible or are uncertain, this model can also be used to estimate or refine parameter values from the toxicity relationships (De Schamphelaere and Janssen, 2002); however, to the extent that model parameters are derived from toxicity data, there is less confidence that the model truly describes the processes regulating bioavailability rather than just providing a reasonable framework for data-fitting.

Despite the successes of this approach for modeling metal bioavailability, certain uncertainties and limitations in the model formulation must be noted:

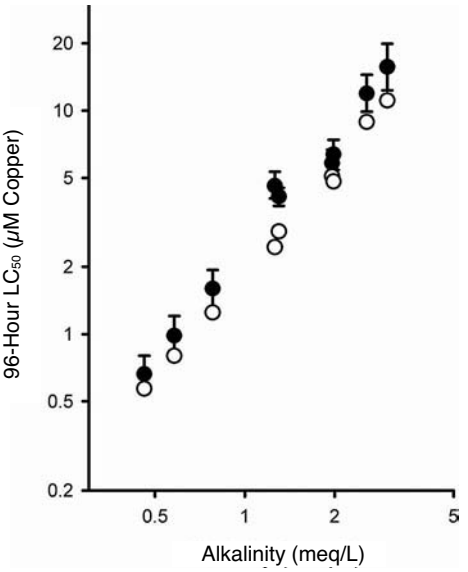

FIGURE 2.12 Observed and predicted lethality of copper to fathead minnows. (Data from Erickson et al., *A Prototype Toxicity Factors Model for Site-Specific Copper Water Quality Criteria*, U.S. Environmental Protection Agency, Duluth, MN, 1987.) Solid circles denote observed LC_{50} values (±95% confidence limits). Open circles denote predicted LC_{50} values using Biotic Ligand Model Version 2.0.0 (Hydroqual; Mahwah, NJ).

1. Most model applications to date do not address effects of changes in chemistry at the gill microenvironment. Tao et al. (2002) reported large effects on apparent metal–gill association constants if the effects of pH changes and mucous secretions on copper binding are accounted for. The implications of this to bioavailability assessments might be significant in some cases and have not been determined.
2. The model assumes chemical equilibria within the exposure water and between the exposure water and the gill surface. In addition, there is a fixed proportionality between the metal bound at the surface and the level of uptake or effect within the organism. Under some circumstances, the kinetics of these processes might be important to bioavailability (Hudson, 1998); for example, speciation changes in the gill microenvironment might not reach equilibrium because of the short residence time of water in the gill.
3. The effects of cations are not necessarily just a matter of competition with the gill sites, as assumed in the model. The effect of hardness on toxicity appears to be nonlinear, whereas the model predicts a linear effect. This might reflect changes in gill permeability that either affect copper bioavailability in a nonlinear fashion or affect toxicity without affecting bioavailability. The amelioration of copper toxicity by sodium should, to some degree, reflect direct effects on sodium exchange rather than competition with copper. Merging this simple bioavailability model with models for ion regulation (Paquin et al., 2002b) would address this issue.
4. The role of copper species other than the free ion in uptake across, or interaction with, the gill surface is poorly established. Efforts to include additional bioavailable species in model calculations (De Schamphelaere and Janssen, 2002) require inferences from toxicity data trends that are difficult, given uncertainties in the data and in model formulation. Better information is needed to establish the actual role of these species.

Interpreting the toxicity of copper and many other cationic toxic metals is difficult because of complex chemical speciation and various other processes that affect their accumulation at, and interaction with, biological receptors. Although the bioavailability modeling approach described here is a rather simple representation of a complex system, it addresses important aspects of the complexity by incorporating

reasonable formulations for some fundamental aspects of metal toxicity: (1) the absorption of bioavailable metal into the organism to achieve toxic metal levels at some site of action, (2) reductions in this absorption due to the formation of less bioavailable metal species in the exposure water, and (3) reductions in this absorption due to the effects of other cations. This section demonstrated how this approach can form the basis for understanding and predicting the role of bioavailability in the effects of exposure conditions on acute copper lethality to fish, as well as how some effects might be related to processes other than bioavailability. The same general considerations will apply to other endpoints, routes of exposure, and metals, although details will vary. Broader and more extensive discussions of metal bioavailability in water can be found in many of the references cited herein. The possible role of dietary metal in the overall bioavailability of metals to fish and other aquatic organisms has been recently reviewed by Meyer et al. (2005). There also is extensive literature concerning metal bioavailability in sediments which, although not directly impacting most fish, is nonetheless important for assessing the broader impacts of metals on aquatic ecosystems (Ankley et al., 1996).

Organometals: Mercury

The behavior of metals in biological systems can change dramatically when they occur as organometallic compounds. Although several metals have the potential to form organometallic compounds, organic species of mercury, tin, and lead have attracted the most attention because of their occurrence in the environment and demonstrated toxicity (Pelletier, 1995). For each of these metals, covalent binding to one or more organic groups yields compounds that may accumulate in fish and other aquatic biota.

A general model for membrane diffusion of trialkyltin compounds was developed in the 1970s (Tosteson and Wieth, 1979; Wieth and Tosteson, 1979). According to the model, positively charged trialkyltin species diffuse across biological membranes in association with an aqueous anion, usually Cl^- or OH^-, and the presence of one or more organic substituents contributes to this uptake by increasing the molecule's relative hydrophobicity, in effect giving it a partially "organic" character. The ion pair model does not, however, account for the fact that organometals tend to retain their metallic (i.e., electrophilic) character, including high reactivity with protein thiols. Transport across a biological membrane is likely, therefore, to reflect a balance between simple diffusion of the neutral ion pair and interactions with membrane proteins (Boudou et al., 1991). Membrane flux of some organometallic compounds may also occur by active transport of complexes formed with small organic molecules (see below).

The dual character of organometals is also reflected in factors that control their speciation in natural waters. As discussed in the previous section regarding copper, pH, alkalinity, and hardness can have a large effect on the speciation, accumulation, and toxicity of inorganic metals. Uptake of organometals also may be impacted to some extent by ionic constituents of water, particularly as they affect the formation of neutral diffusing species. In addition, binding to DOC and POC is likely to be important for controlling the concentration of freely dissolved species, thereby influencing bioavailability in waterborne exposures.

Because organometallic compounds tend to accumulate in organisms that occupy the base of aquatic food webs, the diet may represent the principal route of exposure for higher trophic level organisms, including fish. An understanding of organometal accumulation by fish therefore requires that bioavailability concepts be extended to the entire aquatic food web. Additional consideration must be given to factors that control interconversions of inorganic and organic metal species. This is particularly true when (as is often the case) the concentration of the organometal in fish is referenced to the total concentration of parent metal in sediment or water. In this section, methylmercury accumulation by fish is examined as a means of illustrating these points.

Historically, demonstrated impacts of methylmercury on humans due to consumption of contaminated fish and shellfish have focused attention on point-source releases of mercury to the aquatic environment such as mining, smelting, and wastewater treatment (U.S. EPA, 1997a). Most of the mercury released in these cases exists as elemental or inorganic mercury. Biotic and abiotic processes then transform a portion of this mercury to the methylated form. These releases continue to occur, particularly in countries with emerging industrial economies. Increasingly, however, the focus of mercury research in the United States and elsewhere has been on airborne mercury emissions. Important anthropogenic sources of mercury to the atmosphere include the combustion of fossil fuels, municipal waste incineration, and

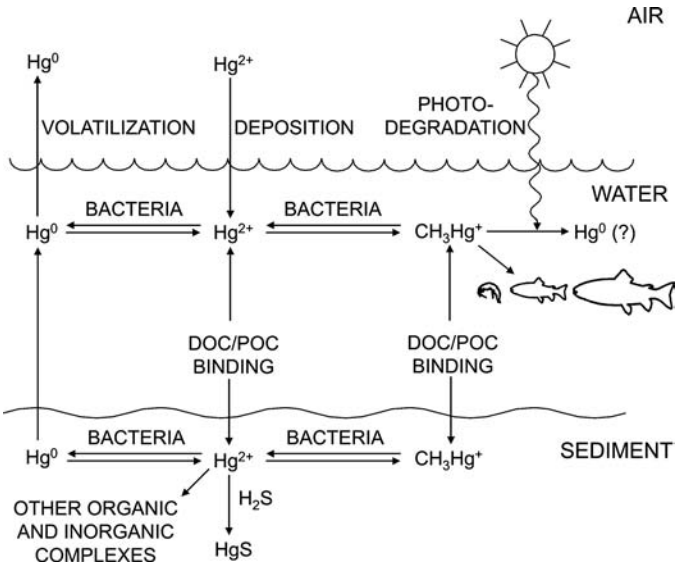

FIGURE 2.13 Biogeochemical cycling of mercury. This figure shows reactions that transform airborne Hg^{2+} into CH_3Hg^+ and the subsequent fate of CH_3Hg^+. Currently, the photodegradation products of CH_3Hg^+ are unknown, although it has been speculated that Hg^0 is produced.

gold mining (also an important point source). To date, 48 states in the United States have issued fish consumption advisories for one or more water bodies because mercury levels in fish muscle tissue exceed state or federal guidelines, and 23 states have issued statewide advisories (U.S. EPA, 2007). Many of these fish reside in water bodies for which there are no known point sources.

Mercury can exist in three valence states:

- Hg^0, metallic mercury (elemental mercury)
- Hg_2^{2+}, monovalent mercury (mercurous mercury)
- Hg^{2+}, divalent mercury (mercuric mercury)

Hg_2^{2+} is unstable under most environmental conditions. The overwhelming percentage of total mercury in the environment therefore occurs as either Hg^0 or Hg^{2+}. Hg^{2+} is commonly associated with sulfur in the mineral cinnebar (HgS) but may complex with other inorganic ligands, including chlorine, oxygen, and hydroxyl ions. Hg^{2+} can also react covalently to form a variety of organic derivatives including CH_3Hg^+ (methylmercury), $(CH_3)_2Hg$ (dimethylmercury), and $C_6H_5Hg^+$ (phenylmercury). Of these, methylmercury (henceforth, MeHg) is by far the most common and is the form of greatest interest to toxicologists due to its high toxicity and propensity to accumulate in aquatic biota (Wiener and Spry, 1996).

The biogeochemical cycling of mercury in freshwater systems has been extensively reviewed (Driscoll et al., 1994; Ullrich et al., 2001; Winfrey and Rudd, 1990; Zillioux et al., 1993). Figure 2.13 shows a subset of known reactions that result in conversions among major mercury species. The same reactions are thought to occur in estuarine and marine environments, but their relative importance is less well known. The focus of this figure is on reactions that transform airborne Hg^{2+} into MeHg, and the subsequent fate of MeHg. Potentially important reactions that do not appear in this figure include the photooxidation of Hg^0 (LaLonde et al., 2001), photoreduction of Hg^{2+} (Amyot et al., 1994), microbial conversion of MeHg to dimethylmercury (Baldi et al., 1993), and formation of soluble sulfide complexes with Hg^{2+} (see below). The percentage of total mercury in freshwater that exists as MeHg varies among systems but is generally <15% and often closer to 5% (U.S. EPA, 1997b). Contaminated sediments serve as an important reservoir for Hg^{2+}, and it is likely that sediment-bound mercury can recycle back into an aquatic ecosystem for many years (Kudo, 1992).

The issue of greatest importance in predicting the environmental fate and effects of mercury is the net rate of methylation (Gilmour and Henry, 1991; Ullrich et al., 2001; Winfrey and Rudd, 1990). Most of the methylation in freshwater systems occurs in sediments, although methylation in the water column has also been described. Anaerobic sulfur-reducing bacteria represent the primary source of MeHg in freshwater systems (Gilmour et al., 1992) and are also active in estuarine (Compeau and Bartha, 1985) and near-shore marine (King et al., 2001) environments. Fulvic and humic material may abiotically methylate mercury, but the mechanism is poorly understood (Weber, 1993). Demethylation is also mediated largely by microorganisms (Robinson and Tuovinen, 1984). Photodegradation of MeHg is known to occur, but the end-products of this reaction and its relative contribution to the mercury cycle are poorly understood (Sellers et al., 1996).

In pore water that contains excess sulfide, dissolved Hg^{2+} complexes with HS^- to form several charged and uncharged sulfide species. Laboratory studies with sulfate-reducing bacteria suggest that the neutral complex HgS^0 can diffuse across bacterial cell membranes and is the principal source of Hg^{2+} for subsequent formation of MeHg (Benoit et al., 1999, 2001). The relative concentration of each sulfide species varies with total sulfide concentration. High sulfide concentrations favor the formation of the charged species $HgHS_2^-$, which is thought to be poorly absorbed by microbes. The addition of sulfate can stimulate methylation in sediment by providing the energy substrate for sulfate-reducing bacteria (Gilmour et al., 1992); however, high levels of sulfate may have a negative impact on mercury methylation due to corresponding increases in sulfide and the resultant removal of mercury from solution as HgS.

Oxides of Fe and Mn may contribute to the formation of MeHg by precipitating Hg^{2+} out of the water column. The formation and dissolution of Fe and Mn oxides are controlled by the redox state and oxygen content of water and sediment. Under the anaerobic conditions often found in sediments, these particles dissolve, making Hg^{2+} available for other reactions. The presence of Fe in sediment can also reduce the inhibitory effect of high sulfide levels on methylation rate, presumably through substrate competition (i.e., the formation of FeS) (Furutani and Rudd, 1980; Gagnon et al., 1996).

Both Hg^{2+} and MeHg accumulate in aquatic biota. Hg^{2+} may predominate at lower trophic levels because it comprises the largest percentage of total mercury in water and sediment. In most instances, however, differences in dietary assimilation and retention of Hg^{2+} and MeHg result in an enrichment of MeHg with each trophic level transfer. The end result is that nearly all of the mercury in fish exists as MeHg (U.S. EPA, 1997b).

By far the largest concentration step for MeHg in the aquatic environment occurs at the base of the food web. Reported BCFs for MeHg in freshwater primary producers (trophic level 1) average about 1×10^5. Methylmercury also biomagnifies in food webs; that is, MeHg concentrations tend to increase at successively higher trophic levels. BMFs for MeHg in freshwater forage fish (concentration at trophic level 3/concentration trophic level 2) and large piscivorous fish (trophic level 4/trophic level 3) range from 2 to about 10 and average around 5 (U.S. EPA, 1997b). Altogether, biomagnification may result in a 20- to 200-fold increase in MeHg concentration from trophic level 1 to trophic level 4. These observations are important because they suggest that factors that control uptake directly from water will determine the amount of MeHg that enters the food web, while those that control uptake from dietary sources will influence the extent to which biomagnification occurs at higher trophic levels.

The dominant form of freely dissolved MeHg in most seawater systems is MeHgCl. Uptake of MeHg by marine phytoplankton was found to be controlled by the concentration of aqueous MeHgCl (Mason et al., 1996). This finding is consistent with earlier work showing that MeHgCl can diffuse readily across artificial lipid membranes (Bienvenue et al., 1984). In freshwater systems, most of the MeHg free in solution exists as MeHgOH. Watras et al. (1998) reported that MeHg concentrations in microseston from 15 freshwater lakes correlated with modeled concentrations of MeHg (free ion) or MeHgOH, but not MeHgCl; however, the diffusion rate for MeHgOH across biological membranes is only 0.04 times that of MeHgCl (Mason et al., 1996). For this reason, Watras et al. (1998) speculated that the uptake of MeHg by microseston was controlled by an active transport mechanism. MeHg complexed with cysteine, thiourea, or thioglycolate was taken up directly from water by sheepshead minnows (Leaner and Mason, 2001). In mammals, MeHg–cysteine complexes are actively transported across placental membranes and the blood–brain barrier by a neutral amino acid carrier system (Kajiwara et al., 1996; Kerper et al., 1992). It is not known whether this or a similar mechanism is active in aquatic biota.

Studies of MeHg accumulation in natural systems are often complicated by the fact that a given factor may impact both bioavailability and net methylation rate. In poorly buffered freshwater systems that are susceptible to acid deposition, MeHg concentrations at all trophic levels tend to be inversely correlated with pH (Watras et al., 1998; Winfrey and Rudd, 1990). Decreased pH, particularly when associated with the deposition of sulfuric acid, has been associated with increased microbial production of MeHg (Gilmour et al., 1991); however, biogeochemical processes that release mercury from soils and sediments at low pH values may also play a role by contributing to the pool of Hg^{2+} available for bacterial methylation. In well-buffered systems and those that drain relatively large watersheds, pH and MeHg accumulation by fish may be poorly correlated (Richardson et al., 1995).

The DOC content of water is an important determinant of MeHg accumulation and, in many systems, a better predictor of fish mercury residues than pH. DOC levels usually correlate positively with MeHg residues in fish, although in a study of Adirondack lakes very high levels of DOC were associated with a decline in MeHg (Driscoll et al., 1995). DOC may contribute indirectly to increased levels of MeHg in fish by promoting the translocation of Hg^{2+} and MeHg from watersheds to water bodies (Hurley et al., 1995). The addition of DOC to a system tends to increase sediment microbial activity, hence the potential for Hg^{2+} methylation. An increase in the DOC content of water may also promote the release of MeHg from sediment to the water column, making it more available for uptake by fish and other aquatic biota (Miskimmen, 1991).

At low Hg^{2+}/DOC ratios, DOC binding of Hg^{2+} is dominated by covalent interactions with thiol groups (Drexel et al., 2002; Haitzer et al., 2002). These complexes are very stable, and it has been suggested that high levels of DOC reduce Hg^{2+} methylation in the water column by reducing uptake of Hg^{2+} by microbes (Gilmour and Henry, 1991). In laboratory studies, DOC addition to water reduced the uptake of Hg^{2+} and MeHg by larvae of a benthic dipteran (*Chaoborus*) (Sjöblom et al., 2000). DOC addition was also shown to reduce MeHg uptake by fish directly from water (Choi et al., 1998).

The affinity of DOC for MeHg and other metals varies with pH. In general, a decrease in pH results in lower MeHg binding to DOC because of competition with hydrogen ion for anionic binding sites (Hintelmann et al., 1995). The binding of MeHg to DOC may also be affected by the presence of other cationic metals. For example, Driscoll et al. (1995) found that MeHg levels in fish were positively correlated with concentrations of monomeric aluminum and suggested that this was because aluminum competes with MeHg for binding sites on DOC, thereby increasing the concentration of unbound MeHg.

In many aquatic systems, benthic organisms contribute substantially to the transfer of energy and contaminants from sediments to the food web. Uptake of Hg^{2+} and MeHg from ingestion of contaminated sediment was investigated by feeding mussels a suspension of sediment particles (Gagnon and Fisher, 1997). Assimilation efficiencies for Hg^{2+} were uniformly low (<10%). Those for MeHg were generally higher (all but one value > 30%) but varied with sediment type. Pretreatment of artificial sediment with organic material (fulvic acid) increased MeHg assimilation in all cases. In contrast, uptake of Hg^{2+} and MeHg from sediment by an estuarine amphipod (*Letocheirus plumulosus*) was negatively correlated with sediment organic matter content, and it was suggested that binding to organic matter prevented MeHg from becoming solubilized within the intestinal tract (Lawrence and Mason, 2001).

Methylmercury levels as a percentage of total mercury are generally very low in sediment but tend to increase in pore water and overlying waters, suggesting that sediment-to-water binding constants for MeHg are lower than for Hg^{2+}. The natural cycling of biological material may also cause MeHg to be retained by organisms living in the water column (Watras et al., 1998). Benthic and pelagic environments may become coupled, however, when sediment-dwelling organisms consume algae or detritus contaminated with MeHg (Lawrence and Mason, 2001) or fish consume benthic species.

Laboratory studies and modeling efforts both indicate that the diet is the primary route of uptake of MeHg for large piscivorous fish (Wiener and Spry, 1996). The predominant route of uptake by smaller fish is less well known, and it has been suggested that uptake from water may contribute substantially to accumulated residues depending on seasonal changes in prey selection and environmental factors (Post et al., 1996). The relative importance of food and water exposure routes in accumulation of MeHg by herbivorous fish and fish that ingest large quantities of sediment is unknown. Depuration studies suggest that the elimination half-life for MeHg in fish may range from weeks to years (Trudel and Rasmussen, 1997). Indeed, elimination proceeds so slowly that MeHg may accumulate throughout a

fish's lifetime, resulting in positive correlations between MeHg concentration and fish age or length (U.S. EPA, 1997a).

Reported assimilation efficiencies for MeHg in dietary uptake studies with fish vary considerably. Several authors have reported dietary assimilation efficiencies ranging from 50 to 90% (Wiener and Spry, 1996), and values in this range have been used successfully in environmental modeling efforts to describe observed rates of MeHg accumulation (Borgmann and Whittle, 1992; Norstrom et al., 1976; Post et al., 1996). In contrast, a low assimilation efficiency (30%) was reported when channel catfish were fed a prepared diet spiked with MeHg (McCloskey et al., 1998), and an even lower value (20%) was obtained when northern pike were fed MeHg-contaminated forage fish (Phillips and Gregory, 1979). This latter finding is particularly interesting because it suggests that the bioavailability of MeHg incorporated into food may be lower than that of MeHg spiked into prepared diets.

The factors that control dietary MeHg uptake by fish are poorly known. Following the digestion of a prey item, much of the MeHg in the gastrointestinal tract probably remains bound to organic compounds and, in particular, to sulfhydryl-containing peptides and amino acids. Although speculative at this time, it is possible that dietary assimilation efficiency may be determined by the extent to which these complexes are actively taken up across the gastrointestinal epithelium.

In summary, the interactions between aquatic organisms and major mercury species are varied and complex. Field surveys have repeatedly shown that MeHg concentrations in large predatory fish from the same general region may differ by a factor of 10 or more. To understand how this can happen we must extend the concept of bioavailability to consider factors that control MeHg production as well as its uptake and accumulation in aquatic organisms. Three critical interactions are thought to control MeHg production and accumulation in a wide range of systems. The first of these occurs in anaerobic sediments and involves the uptake of Hg^{2+} by sulfate-reducing bacteria. Recent work suggests that in the presence of sulfide this uptake is controlled by the formation of a neutral sulfide complex. The second interaction involves the uptake of MeHg by lower trophic level organisms living in the water column. In saltwater systems, this uptake is probably dominated by simple diffusion of neutral MeHgCl, while in freshwater systems the diffusing species is more likely to be MeHgOH. Strong binding to DOC limits the amount of MeHg available to form these complexes. Other modes of uptake into lower trophic level organisms (e.g., active transport of MeHg complexes) have also been proposed. The third interaction involves dietary uptake of MeHg by fish. The identity of the chemical species that is absorbed within the gastrointestinal tract is unknown but may include MeHg complexes with small organic molecules.

Nonionic Organics: 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) provides an excellent example of how bioavailabilities of persistent, hydrophobic, nonpolar organic chemicals influence exposures and consequent toxicity risks to aquatic organisms. With a molecular weight of 322, a log K_{ow} of approximately 7, and a planar molecular structure, TCDD strongly associates with organic carbon, whether it be in soils, sediments, POC, or DOC. The hydrophobic character of TCDD not only thermodynamically drives its partitioning in the abiotic environment but also is responsible for a strong bioaccumulation/biomagnification potential which causes TCDD to accumulate in the lipid-containing tissues of biota in food webs. In most risk assessments concerned with the toxicity of TCDD, it is now recognized that the combined contributions of all chemicals with a TCDD-like mode of action to overall risk must be evaluated (Van den Berg et al., 1998). The identification of chemicals that act additively with TCDD is based on their persistence in the environment and ability to act through an aryl hydrocarbon receptor (AhR)-mediated mechanism of action as does TCDD. Typically, the contribution of specific congeners with polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl structures are considered. Thus, differences between these congeners and TCDD, in bioavailability as well as relative potencies, are important in quantitative risk assessments for complex mixtures of these chemicals which commonly occur in the environment. The retrospective assessment of AHR-mediated toxicity to lake trout sac fry in Lake Ontario provides a case study that validates this predictive capability (Cook et al., 2003).

The bioavailability of TCDD to fish involves both the amount of TCDD available for direct uptake by fish from water and sediment and the amount of TCDD in their food, which is influenced by the

bioavailability of TCDD to organisms in the food chain. Theoretically, more organic carbon in the water and sediments of an aquatic ecosystem should result in reduced bioavailability of TCDD to fish. A consequence of low amounts of organic carbon in an aquatic ecosystem would be increased bioavailability and thus bioaccumulation of TCDD.

In water, the bioavailability of a nonpolar, hydrophobic organic chemical is proportional to the fraction of chemical that is freely dissolved (f_{fd}), which is related to the concentrations of POC and DOC as follows:

$$f_{fd} = \frac{1}{1 + (POC)\left(K_{poc}\right) + (DOC)\left(K_{doc}\right)} \approx \frac{1}{1 + (POC + DOC/10)\left(K_{ow}\right)} \tag{2.8}$$

where organic carbon partition coefficients for POC (K_{poc}) and DOC (K_{doc}) can be approximated as K_{ow} and $K_{ow}/10$, respectively (Eadie et al., 1992). Slightly different values of K_{poc} are commonly used based on laboratory studies, such as those of Karickhoff (1981), which determine organic carbon partition coefficients for sediment organic carbon (K_{soc}) from sorption/desorption rates for sediment rather than suspended solids. As a first approximation, one may assume $K_{poc} \approx K_{soc}$. In the past decade, considerable debate occurred among environmental chemists concerning the nature of organic chemical binding to particles in water and the degree to which equilibrium models for the distribution between water and organic carbon on particles are appropriate for modeling hydrophobic organic chemical distribution in natural systems (Schrap and Opperhuizen, 1992). Measurement of K_{poc} or K_{soc} for TCDD is complicated by the inability to conduct experiments with no organic carbon in the water. Extrapolation of TCDD sediment desorption measurements to zero aqueous organic carbon resulted in an estimate of log K_{soc} of 7.25 to 7.59 (Lodge and Cook, 1989), a value significantly greater than the estimated log K_{ow}. Aside from the possibility that log K_{ow} has been underestimated for TCDD, this discrepancy might be due to higher affinities of planar aromatic compounds such as TCDD for *black carbon** in sediments (Barring et al., 2002) than for other sediment carbon, for which TCDD partitioning more closely follows K_{ow}.

The degree to which values of K_{doc} are less than K_{poc} or K_{soc} is also uncertain and may vary with the chemical composition of dissolved organic carbon in different ecosystems (Burkhard, 2000; Chin and Gschwend, 1992; Eadie et al., 1990). Clearly, accuracy in the prediction of concentrations of freely dissolved TCDD in different waters depends heavily on the accuracy of the determination of K_{ow} for TCDD. Figure 2.14 demonstrates how, under different POC conditions, f_{fd} varies for TCDD with an estimated log K_{ow} of 7 vs. an estimated log K_{ow} of 8. A major impediment to the direct determination of concentrations of freely dissolved TCDD in water is the difficulty of separating freely dissolved TCDD from bound TCDD in water samples, combined with the challenge of detecting and measuring concentrations on the order of femtograms/liter (parts per quintillion).

Because a major fraction of TCDD in aquatic systems is associated with organic carbon, exposure of aquatic organisms to TCDD and the resulting bioaccumulation depend greatly on each organism's connectivity to sediments or suspended particles. Benthic organisms have direct contact with sediment and accumulate hydrophobic aromatic chemicals to a greater extent through ingestion of sediment than from exposure to pore water (Leppänen and Kukkonen, 1998). If the concentration of TCDD varies with depth in the sediment, the depth to which the organism burrows in the sediment will influence exposure. Ingestion of TCDD with food by oligochaetes (*Lumbriculus variegatus*) and midges (*Chironomus tentans*) results in accumulation of TCDD in the organisms at levels close to equilibrium with the food (West et al., 1997). Slow rates of elimination from these benthic invertebrates suggest an inability to metabolize TCDD. Bioaccumulation of organic chemicals with log K_{ow} values greater than 5.5 by pelagic fish can be strongly influenced by the presence of a benthic food chain component in the diet (Burkhard,

* *Black carbon* refers to carbon from a variety of sources such as combustion particles (soot), fly ash, coal dust, and tire dust. The different forms of black carbon can have different partitioning properties than carbon originating from low-temperature biogenic processes. For sediments, the term *organic carbon* includes both biogenic and black carbon, in contrast to inorganic forms of carbon such as carbonates. Black carbon usually comprises a small fraction of the total organic carbon measured yet has the potential to exert a greater relative influence on partitioning and bioavailability of planar organic chemicals such as TCDD and BaP.

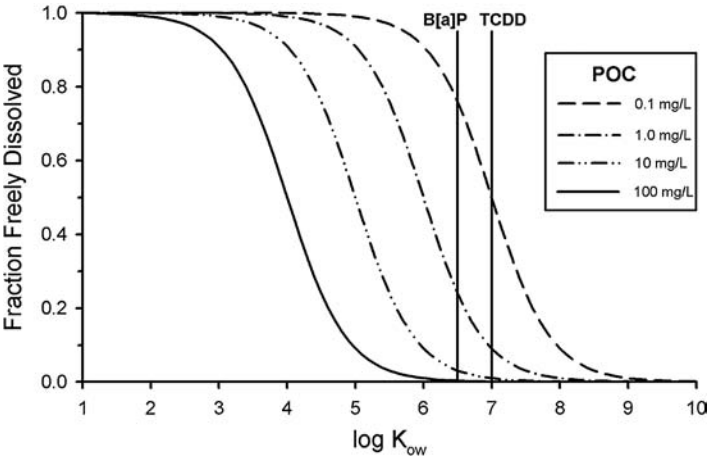

FIGURE 2.14 Effect of particulate organic carbon (POC) concentrations and octanol–water partition coefficient (K_{ow}) on the fraction of freely dissolved chemical. Vertical lines intersect expected fractions at different POC concentrations for benzo(*a*)pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

1998); thus, the high degree of bioavailability of TCDD and related chemicals from sediment to benthic invertebrates is often passed on to fish and wildlife through their diet.

Bioaccumulation of TCDD by epibenthic invertebrate or benthic vertebrate organisms often may not attain equilibrium with sediments due to disequilibrium between sediments and overlying water. The concentration of the chemical in the overlying water influences not only uptake through respiration and dermal absorption but also dietary uptake, because concentrations in some food items at the sediment–water interface are more related to water than sediment. The extent of this disequilibrium can be expressed by the variable:

$$\prod_{socw} = \frac{C_{soc}}{C_w^{fd}} \tag{2.9}$$

where C_{soc} is the organic-carbon-normalized chemical concentration in the sediment, and C_w^{fd} is the freely dissolved concentration in the overlying water. At equilibrium, $\prod_{socw}$ should approximately equal K_{ow}, so the ratio $\prod_{socw}/ K_{ow}$ indicates the degree of disequilibrium between sediment and overlying water (Cook and Burkhard, 1998). Field sampling as well as dynamic chemical mass balance models indicate that $\prod_{socw}/K_{ow}$ for TCDD and other hydrophobic organic chemicals is often greater than 1.0 (Burkhard et al., 2003). This disequilibrium appears to be largely attributable to two factors: (1) slow mixing of surface sediment with and diffusion of TCDD to overlying water when concentrations of TCDD in sediments reflect greater chemical loadings to the system in the past, and (2) the presence of greater amounts of biogenic organic carbon in water particulates than in sediment, which causes the fraction of freely dissolved chemical in the overlying water to be less than in sediment pore water. Difficulty in measuring C_w^{fd} and K_{ow} for very hydrophobic chemicals such as TCDD can complicate direct determinations of $\prod_{socw}/K_{ow}$. On the other hand, it often is not difficult to semiquantitatively determine this ratio with knowledge of the hydrodynamic characteristics of the aquatic ecosystem and the relative chemical loading rate over time.

The bioavailability of TCDD and related chemicals is not just a function of their hydrophobicity and how this affects environmental partitioning and uptake via water and food. Bioaccumulation of these chemicals by vertebrates is reduced by biotransformation, which increases the rate of elimination. Chlorinated dioxins and furans are eliminated faster by fish than PCBs with similar log K_{ow} values (Van der Linde et al., 2001). Biotransformation of TCDD is specifically indicated by measurement of TCDD metabolites in the bile of rainbow trout (Kleeman et al., 1986a) and yellow perch (Kleeman et al., 1986b). The rate of elimination of a chemical similar to TCDD, 2,3,4,7,8-pentachlorodibenzofuran, from rainbow

trout is reduced by the metabolic inhibitor piperonylbutoxide (Sijm et al., 1990). In aquatic food webs, TCDD does not appear to biomagnify in fish as do PCBs with similar K_{ow} values. Because dietary assimilation efficiencies of TCDD are high (Isosaari et al., 2004; Nichols et al., 1998), this difference between TCDD and PCBs cannot be attributed to lower dietary bioavailability for TCDD than PCBs, but rather is likely due to biotransformation in both prey and predator species of fish (Burkhard et al., 2004; Endicott and Cook, 1994).

This discussion of TCDD illustrated how the bioavailability of hydrophobic organic chemicals is strongly influenced by their partitioning to organic carbon in aquatic systems. The freely dissolved (most bioavailable) fraction of such chemicals is reduced by DOC and POC, and much of the chemical is partitioned into sediments. The importance of dietary exposure increases with chemical hydrophobicity, especially for organisms at higher trophic levels whose dietary exposures can be affected by biomagnification through the food chain. Bioavailability for such organisms becomes a function, not just of their exposure to water and food but also of the bioavailability relationships for their entire food chain. Under these circumstances, chemical accumulation in fish is determined by both chemical speciation in the water and in the sediments associated with the benthic-based part of the food web. These processes have been addressed in food-web-based bioaccumulation models that can assess the bioavailability to fish of hydrophobic organic contaminants throughout entire aquatic systems (Burkhard et al., 2003). The next case study considers a chemical in which biotransformation is even more important for determining bioavailability.

Nonionic Organics: Benzo(*a*)pyrene

Benzo(*a*)pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) with a planar structure composed of five fused benzene rings, a molecular weight of 252.3 Daltons, and log K_{ow} of 6.5 (Bruggeman et al., 1982). Unlike TCDD, BaP concentrations in most surficial sediments have not declined significantly since the 1970s (Lima et al., 2003). BaP also provides an interesting contrast to TCDD with regard to bioavailability. Both chemicals have gained notoriety because of their potential carcinogenicity, but, although only slightly less hydrophobic, BaP appears to be much less bioaccumulative in fish than TCDD. Fish BSAFs for PAHs in the environment are about three orders of magnitude less than for chlorinated aromatic hydrocarbons (Connor, 1984). For more extensive reviews of PAH bioaccumulation in marine organisms, see Meador et al. (1995).

One factor responsible for lower bioaccumulation of BaP in fish than for TCDD appears to be a much greater affinity of BaP for organic carbon, despite these two chemicals having similar molecular weights and hydrophobicities. As noted in the TCDD case study above, partition coefficients of organic chemicals to DOC are typically about tenfold less than the K_{ow} for the chemical; however, the binding of BaP with dissolved humic material was found to be completely reversible with a partition coefficient approximately equal to its K_{ow} (McCarthy and Jimenez, 1985). For POC, partition coefficients for Baltic Sea water (Broman et al., 1991) were 20-fold greater for BaP than for TCDD, which is slightly more hydrophobic.

This low availability of BaP in water is also evident in sediments, which is consistent with the low bioaccumulation observed in fish dependent on benthic food chains. In general, PAHs in sediments seem to be less bioavailable to benthic invertebrates than other persistent organic chemicals. Bioaccumulation of PAHs by various benthic-coupled organisms in both field and laboratory exposures is an order of magnitude less than for PCBs or pesticides, which, unlike the PAHs, tend to reach equilibrium distributions between sediment organic carbon and organism lipid (Tracey and Hansen, 1996). This low bioavailability of PAHs may be attributable to lower concentrations of PAHs in pore water than expected based on their K_{ow} and their total concentrations in the sediment. The uptake of chemicals by benthic organisms, however, is not just via pore water. Ingestion of sediment appears to be an important route for accumulation of PAHs from sediment by oligochaetes (Leppanen and Kukkonen, 1998). The greater uptake of BaP from ingested sediment particles by an oligochaete in comparison to the less hydrophobic PAH phenanthrene is attributable to the greater assimilation efficiency of BaP which causes a greater uptake from the ingested particles than from pore water (Lu et al., 2004). For such organisms, the overall bioavailability of BaP from sediments will not be as restricted as suggested by the partitioning between water and sediment.

One complication that impacts the bioavailability of BaP and other pyrogenic PAHs in aquatic systems is an association with the black carbon phase in sediments and suspended particles. Because BaP is derived from combustion reactions, it may largely be transported through the atmosphere to aquatic systems in fine soot particles to which it is more strongly bound than to biogenic organic carbon in sediment. Thus, K_{oc} values measured for ambient environmental conditions may be significantly greater than predicted from K_{ow} because the black carbon component of sediments significantly reduces the amount of BaP in the sediment available for desorption to the water phase. Activated carbon–water partition coefficients have been suggested for prediction of the effect of black carbon in sediments on PAH concentrations in pore water (Gustafsson et al., 1997). This requires that the fraction of black carbon in the sediment be determined in addition to the fraction of biogenic organic carbon. Although PCBs were observed to be 100% available for equilibrium partitioning to water from sediments in desorption experiments, only a small fraction of pyrogenic PAHs in the sediments were similarly available (McGroddy et al., 1996). This partitioning difference is probably related to whether the chemical is sorbed on the surface (available for desorption) vs. incorporated into the particle matrix (less available for desorption). The degree to which a chemical formed during a combustion process is locked into the matrices of fine combustion particles probably depends on the volatility of the chemical, the combustion temperature, and the gas/particulate emission conditions.

Another factor that may limit benthic food chain transfer of BaP to fish is biotransformation in benthic invertebrates at the base of the food chain. Two species of deposit-feeding marine amphipods (*Rhepoxynius abronius* and *Eohaustorius washingtonianus*) were found to extensively metabolize BaP accumulated from sediment into three different BaP–diol and two hydroxy–BaP molecules (Reichert et al., 1985). Similarly, there was evidence of formation of phase I and phase II metabolites of BaP accumulated from sediment by larvae of a midge (*Chironomus riparius*) and a fingernail clam (*Spaerium corneum*), although much of the BaP remained unmetabolized after 5 days following a 5-day exposure (Borchert et al., 1997). Shorter exposures of *C. riparius* to BaP in water seemed to result in faster rates of metabolism and elimination (Leversee et al., 1982). This difference could be related to an inability to reach an internal steady-state distribution of BaP in shorter exposures. Three species of polychaete worms exposed for 8 days to BaP on sediment showed different rates of metabolism, with half-lives for elimination ranging from 3.7 to 10.3 days (Driscoll and McElroy, 1996). The fraction of BaP identified as metabolites in ten small invertebrate species ranged from 7 to 96% (McElroy et al., 2000). Despite this metabolism in invertebrates, fish can still receive significant PAH exposure from the consumption of benthic invertebrates. Zebra mussels (*Dreissena polymorpha*) in the Detroit River and western Lake Erie were found to accumulate BaP to concentrations up to 8 ng/g. Freshwater drum (*Aplodinotus grunniens*) from this area had large concentrations of PAH metabolites in their bile, indicating extensive exposure to PAHs (Metcalfe et al., 1997). The stomach contents of bottom-feeding fish have been found to contain substantial concentrations of PAHs, including BaP (Maccubbin et al., 1985). The bioavailability of BaP to fish is complicated by the degree to which metabolism affects rates of BaP elimination in organisms throughout aquatic food webs and the possibility of direct exposure through ingestion of sediment.

Accumulation of PAHs in tissues within fish is further limited by biotransformation by the fish. Reports of BaP metabolism in fish trace back at least to 1972 (Lee et al., 1972). The BCF for unmetabolized BaP in bluegill sunfish (*Lepomis macrochirus*) was determined to be 490, which was about 60 times less than that predicted from the K_{ow} (Spacie et al., 1983). The BCF measured for unmetabolized BaP in gizzard shad (*Dorosoma cepedianum*) after a 3-day exposure via water was only 3.2, but preexposure to the fungicide clotrimazole, which inhibits P450 activity, increased the BCF to 35 (Levine et al., 1997), illustrating the potential for chemical interactions that might influence BaP metabolism under environmental exposure conditions. BaP is extensively metabolized in fish livers, so unmetabolized BaP is usually not detectable in liver tissue (Varanasi and Gmur, 1981). In fact, the distribution of BaP and metabolites in northern pike (*Esox lucius*) tissues, following water exposure for 8.5 days with [^{3}H]-BaP, was dominated by the disposition of metabolites, with 15, 40, and 1300 times more radioactivity in liver, kidney, and gallbladder, respectively, than in adipose tissue (Balk et al., 1984). Temperature was shown to influence both the rate of metabolism and the composition of the metabolite mixture produced by the gulf toadfish (*Opsanus beta*) (Kennedy et al., 1989). Dietary exposure of fish to BaP results in metabolism

in the intestine, so BaP absorbed from food enters circulation with its metabolites (Van Veld et al., 1988). When bluegill sunfish during aqueous exposure to BaP were fed uncontaminated food, the rate of elimination of BaP and metabolites was ten times greater than with no food, and the rate of conversion of BaP to polar metabolites was also increased (Jimenez et al., 1987). This suggests that having food in the gastrointestinal tract, in addition to increasing elimination of BaP by partitioning to feces, facilitates the exchange of BaP from blood to the gastrointestinal mucosa where metabolism can accelerate elimination through the digestive tract.

The role of metabolism in limiting the bioavailability of BaP to fish, and thus risk to fish populations, is uncertain because this has been investigated mainly for juvenile and adult fish, not for the embryo–larval stages, which are likely more sensitive. The bioavailability of BaP to fish early life stages may be particularly critical for determination of the phototoxicity risks of PAHs such as BaP because larvae appear to be especially sensitive for such toxicity (Oris and Giesy, 1987). Some studies have indicated that BaP is more bioaccumulative in the early life stages of fish than in older stages. Comparison of BCFs for PCBs and PAHs provides an effective measure of the degree to which fish metabolize PAHs. The lipid-normalized, steady-state BCFs for BaP (measured as total [^{14}C]-BaP) and PCB 105 in zebrafish (*Brachydanio rerio*) larvae were found to be approximately equal to K_{ow}, suggesting a lack of metabolism of BaP during the 7 days of exposure (Petersen and Kristensen, 1998). Similar results were found for other PAHs tested with larvae of three other fish species; however, the retention of [^{14}C] from labeled BaP in fish embryos does not necessarily indicate the absence of metabolites, as clearance of [^{14}C]-labeled metabolites from an embryo may be slow. Exposure of trout embryos to BaP in water/DMSO resulted in retention at hatch of 60 to 80% of the initial concentration in the egg, with indications that some metabolites were being excreted (Kocan and Landolt, 1984). Confocal microscopy more recently revealed that both BaP and its metabolites remained in water-exposed medaka embryos during very early development (Hornung et al., 2005). A further complication is that PAH mixtures consist of both inducers and inhibitors of CYP1A activity, and inhibition of CYP1A in fish embryos has been observed to increase the embryotoxicity of BaP in mummichog (*Fundulus heteroclitus*) (Wassenburg and Di Guilio, 2004).

This section has examined two factors that distinguish the bioavailability of BaP from TCDD and should be considered in the assessment of the bioavailability of any hydrophobic organic chemical. First, the strength of the association of a chemical with organic carbon can deviate from simple correlations to hydrophobicity measures such as K_{ow}. This can be true for reversible adsorption to or partitioning into organic phases but is especially important for systems contaminated with combustion products, in which a portion of some chemicals might have low bioavailability due to occlusion within particles. Second, biotransformation of PAHs both within the food chain and the receptor fish can greatly reduce bioaccumulation within the fish compared to that expected in the absence of such metabolism. For hydrophobic chemicals with slow rates of elimination, even slow rates of metabolism can contribute substantially to overall elimination and thus to steady-state bioaccumulation. The impact of biotransformation will vary with the species and lifestage of the organism of concern. Both of these factors involve various components in a food web, and the resultant bioavailability to a fish must be assessed in terms of the entire web and its spatial extent.

Summary

The bioavailability of chemical contaminants to fish can vary greatly among chemicals, organisms, and exposure conditions, so it is therefore of great importance to fish toxicology and risk assessments. Although bioavailability is a conceptually simple concept—addressing the amount of chemical accumulation by an organism relative to an environmental expousure—it is often difficult to assess and apply. Bioavailability assessments must start with a clear definition of the risk situation of interest. Across what exposure conditions are bioavailability comparisons being made? What organisms and chemicals are of concern? What environmental concentrations and what concentrations within an organism are the references for defining bioavailability? What are the spatial and time frameworks? What exposure pathways should be considered? Chemical speciation is a primary focus for understanding and describing

bioavailability. Bioavailability assessments must address the various chemical species to which an organism is exposed and how readily each of these species is absorbed into the organism. Sometimes, assessments must also address how these species are formed and reach the organism of interest and how they are processed by the organism after absorption prior to reaching a site of action. This involves consideration of a variety of processes in the organism's environment, at the interface between the organism and its environment, and within the organism. The materials presented in this chapter exemplify the issues and approaches that have to be considered when developing bioavailability assessments for specific problems, to ensure that the assessments adequately address the relationship of accumulation to environmental exposures.

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3

Toxicokinetics in Fishes

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CONTENTS

Introduction

Fish are intimately linked to their aqueous habitat. They respire, osmoregulate, achieve acid–base balance, and obtain their thermal character in relation to the surrounding water. In addition, water serves as a common conduit for many essential life processes. Most fish eat, drink, urinate, defecate, swim, obtain sensory information, reproduce, and spatially orient within a single surrounding and contiguous medium. The structural and physiological adaptations that allow fish to thrive in an aqueous environment also strongly impact their interactions with xenobiotic substances. The solvent properties of water and its contrasting character relative to many important contaminants further shape these interactions.

Absorption, distribution, biotransformation, and excretion of xenobiotics by fish are important determinants of chemical toxicity. These processes influence whole-organism dose–response relationships by altering the effective concentration over time at a target site. The degree to which any individual process alters the concentration and activity of a toxicant depends on the character of the toxicant and attributes of the exposed organism. The role that individual organs play in each process and the molecular susceptibility of specific tissues and organs provide the basis for specific toxicities.

Most of the literature dealing with the movement of chemicals into, through, and out of living systems addresses drugs and their behavior. The term *pharmacokinetics* is used to describe the study of such movements, particularly as they vary in time. *Toxicokinetics* deals with the movement of toxicants or compounds that are present at toxic concentrations. This distinction aside, the two terms are synonymous; thus, *toxicokinetics* refers to the study of time-dependent absorption, distribution, biotransformation, and excretion of toxic substances with the goal of providing a description of the chemical concentration time course at the site of action. Most of the toxicokinetic studies published to date have been conducted using well-known mammalian laboratory species. Although they utilize many of the same basic tenets employed in these mammalian efforts, studies with fish may require customized mathematical modeling formats and experimental approaches due to structural, physiological, and lifestyle differences.

The processes of absorption, distribution, biotransformation, and excretion also determine the extent to which xenobiotics bioconcentrate and bioaccumulate in fish. *Bioconcentration* refers to the uptake and accumulation of chemicals directly from water. *Bioaccumulation* is a more inclusive term that describes chemical uptake and accumulation from all sources: water, diet, and sediments. Bioconcentration and bioaccumulation, particularly of persistent compounds, serve as indicators of past exposure for the animal; as indicators of potential exposure for higher trophic levels, including the human consumer; and as markers of potential toxicity.

This chapter examines the topic of toxicokinetics in fish by first reviewing the anatomical, physiological, and environmental determinants of chemical uptake and disposition. The goal of this chapter is to link these structural and physiological determinants with empirical data and kinetic modeling approaches that describe this composite behavior.

Basic Concepts

Fish as Wet Vertebrates

The basic requirements for life are the same for fish as other organisms. Life in water, however, presents a number of challenges to fish that are not equitably shared by most terrestrial animals. Water has a high specific heat capacity, which makes it impossible for all but the largest fish to maintain internal temperatures different from those of the immediate surroundings. Compared with air, water contains low levels of dissolved gases. The solubilities of physiologically important gases in water also differ. These facts necessitate both specialized gas-exchange surfaces and a different approach to acid–base balance. The viscosity of water requires that fish employ specialized approaches to locomotion and expend considerable energy to support respiration. A number of the adaptive physiological and structural modifications that these challenges elicit either directly or indirectly influence xenobiotic toxicokinetics. As an example, poikilothermy influences membrane structure and function, enzymatic isozyme profiles, cardiac output, bile flow rate, and the intestinal passage rate.

Although the mechanisms available to fish for homeostasis are similar to those of other vertebrates, the ways that they are used may or may not be the same. This is important to keep in mind when comparing the kinetics of xenobiotics between fish and mammals, among fishes of diverse groups, and even among individuals of the same species held under different environmental conditions. Table 3.1 lists some generalized structural and functional differences between fish and mammals (Cunningham, 1997; Evans, 1993; Ruckesbusch et al., 1991). It will become clear through the course of this chapter that many of these differences influence xenobiotic absorption, distribution, and elimination in fishes.

Uptake of xenobiotics by fish may occur by inhalation, ingestion, or dermal exposure, as is the case for their mammalian counterparts (Figure 3.1). Likewise, the excretion of xenobiotics by fish and mammals occurs by diffusion or transport across respiratory and skin surfaces, as well as by urinary, biliary, and fecal routes. Even with these commonalities, there are some obvious and not so obvious differences between fish and mammals with regard to the nature of these pathways, the relative importance of each pathway, and xenobiotic movements at the exchange surfaces that separate the animal from its environment.

Two of the more obvious differences between fish and mammals are that fish live in water and respire using gills. The fact that the surrounding medium is water rather than air strongly influences the nature of chemicals delivered to and eliminated at the gills. The mammalian lung, for example, is exposed primarily to volatile chemicals and chemicals associated with or existing as particulates. In contrast, the gill is commonly exposed to water-soluble chemicals, as well as nonpolar compounds adsorbed to dissolved and particulate organic matter. Branchial uptake requires diffusion across the gill membranes, which possess a hydrophobic core. For nonpolar compounds bound to organic matter in water, uptake requires first that they dissociate from the organic material and traverse a polar water and mucus interface before diffusing across a compatible hydrophobic membrane. The same phase transitions exist for chemical excretion across the gills. In general, the gills play a much greater role in the absorption and excretion of xenobiotics by fish than the lung plays in mammals.

The skin of mammals is dry, dead, and keratinized and does not possess a circulatory component. When present, fur may further limit dermal uptake of xenobiotics in mammals. In contrast, the skin of fishes is a living epidermis that is perfused by an underlying secondary circulation. In very small fish or benthic species that are in constant contact with contaminated sediment, the skin becomes an important exchange surface for xenobiotic compounds. The solvent fluid nature of water, combined with the foregoing considerations, suggests that this route of exposure is more important for these fish than for most mammals.

TABLE 3.1

Generalized Structural, Functional, and Environmental Differences between Fish and Mammals

Characteristic	Fish	Mammals
Media		
Surrounding media	Water	Air
Specific heat	High	Low
Solvent properties	Universal solvent	Nonsolvent
Dissolved gases	Low levels	High levels
Viscosity	High	Low
Temperature		
Modality	Poilkilotherms	Homeotherms
Metabolic rate	Slow	Fast
Primary energy currency	Amino acids and lipid	Carbohydrates
Feed/weight conversion	Very efficient	Less efficient
Membrane composition	Homeoviscous adaptation	No adaptation necessary
Body temperature regulation	Behavioral	Internal setpoint
Respiratory surface		
Primary organ	Gill	Lung
Blood/gas flows	Counter-current	Directional/tidal flow
Gas exchange	Yes	Yes
Acid–base balance	Yes	Yes
Nitrogen excretion	Yes	No
Osmoregulation	Yes	No
Primary driver of respiration	Low oxygen	High carbon dioxide
Acid–base balance		
Primary organ	Gill	Lung and kidney
Primary mechanism	Ion based	Gas (lung) and ion (kidney) based
Nitrogenous waste elimination		
Primary organ	Gill	Kidney
Primary form	Ammonia	Urea
Circulation		
Heart	Two chambers	Four chambers
Arrangement	Heart and gill in series	Heart and lung in parallel
Vessels	Arteries and veins less distinct	Distinct arteries and veins

The gastrointestinal tract plays an important role in the absorption of xenobiotics in both fish and mammals. The structure and function of the gastrointestinal tract in both taxa are similar in many respects. In fish, however, the absence of a lymphatic system and lack of classical mammalian villi may substantially influence the absorptive process. The feces represent an important route of elimination in fish, both as a purveyor of bile and as a matrix with affinity for exsorbed compounds. Although bile formation is slow in fishes relative to mammals, many xenobiotic compounds are readily excreted in bile.

In most cases, urinary elimination of xenobiotics is less important in fish than it is for mammals. This is due in part to the contribution of the gills to chemical elimination and to the modified role of the kidneys in comparison with mammals. In other cases, however, the urine may represent the primary route of elimination. Urinary elimination is particularly important for ionic compounds that act as substrates for active transport systems within the renal tubular epithelium.

The Environment

Large differences often exist among environmental toxicant concentrations, the concentration in the systemic circulation of the fish, and the concentration involved in specific target organ toxicity. The basis for these differences resides with both environmental factors and factors associated with the fish. The amount of chemical released to the environment, its movement among compartments (e.g., air, water, sediment), and susceptibility to transformation are primary determinants of toxicant exposure to the

TABLE 3.1 (cont.)

Generalized Structural, Functional, and Environmental Differences between Fish and Mammals

Characteristic	Fish	Mammals
Lymphatics	No	Yes
Secondary circulation	Yes	No
Blood pressure and flow	Low	High
Red blood cells	Nucleated	Non-nucleated
Plasma proteins	Lower levels	Higher levels
Blood pH	Higher that mammals	Lower than fish
Kidney		
Nephron	Less complex. SW—no distal tubule; aglomerular. FW, SW—modified loop of Henle	More complex, including glomerulus, distal tubule and loop of Henle
Blood supply	Renal artery and renal portal	Renal artery
Hematopoiesis	Many fishes	NA
Waste excretion	Minor site	Primary site
Fluid balance	Primary site	Primary site
Ion balance	Secondary site	Primary site
Acid–base balance	Secondary site	Primary site
Liver		
Functional unit	Tubule, with no triads or zonal relationships	Lobule, with triads and zonal arrangement
Gallbladder	May or may not be present	May or may not be present
Bile flow	Slower than mammals	Faster than fish
Biotransformation rates	Slower than mammals	Faster than fish
Gastrointestinal tract		
Structural divisions	Gradual transitions	Clear delineations
Gastric stomach	Absent in 15% of species	Present
Absorptive surface	Longitudinal folds	Villi
Lipid absorption	Primarily into blood	Size dependent into lymph
Change in temperature	Change in length/morphology	NA
Skin		
Epidermis	Living, moist	Dead, dry, keritinized
Mucous	Present	Absent

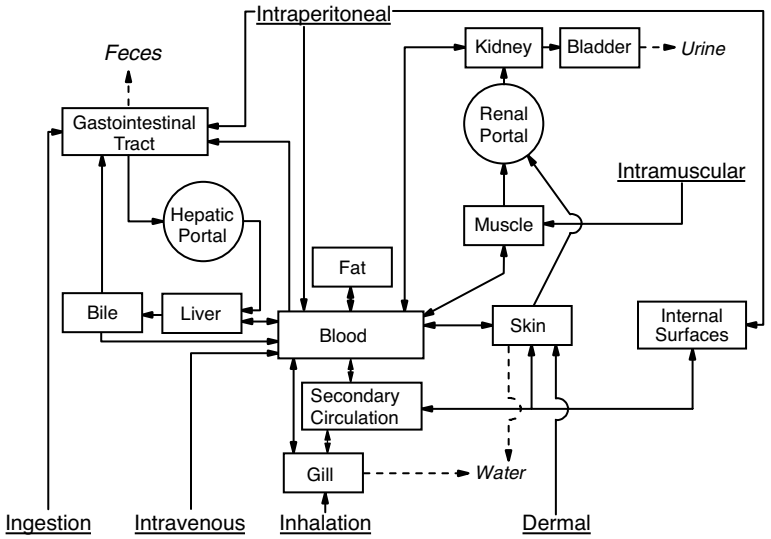

FIGURE 3.1 Pathways for absorption, distribution, and elimination of xenobiotic compounds in fish.

exchange surfaces of fish. Conceptually similar processes operate within the fish, modulating exposure to the sites of toxicity.

Water dissolves more substances and dissolves them more completely than any other liquid; nevertheless, some nonpolar substances are relatively insoluble in water. This solubility or insolubility is a primary determinant of the distribution and availability of contaminants to aquatic organisms. In general, polar compounds that are nonvolatile will remain freely dissolved in the water column. Alternatively, if the compound is nonpolar and nonvolatile, it will partition to organic matrices in the water, reducing the amount that is freely dissolved. In doing so, these chemicals may exhibit an apportioning equilibrium that reflects their relative polarity. These characteristics influence not only toxicant availability to the fish but also the primary routes of uptake.

Water in natural systems is a composite of H_2O and the materials dissolved and suspended in it. Because these materials influence the complexation, binding, precipitation, and chemical form of many compounds, water cannot be viewed as a uniform or generic medium. Water quality as it relates to aquatic toxicology includes both biotic and abiotic components. Living organisms and organically derived substances contribute to the biotic character of water. Abiotic factors include pH, hardness, alkalinity, salinity, dissolved solids, and temperature. Collectively, water quality can influence the chemical form, availability, and ultimately toxicity of chemicals in the aquatic environment. Often the influence of water quality on toxicity is a collective action, with each characteristic exerting independent as well as interdependent effects. A comprehensive discussion of chemical bioavailability, focusing on environmental processes that control chemical uptake and accumulation by fish, is presented in Chapter 2.

Membranes and Xenobiotic Movement

Xenobiotic absorption, distribution, and elimination are dependent on transport across one or more biological membranes. All known cellular membranes are composed of lipid bilayers arranged with hydrophilic polar regions facing the outer surfaces and hydrophobic regions oriented toward the interior. On either surface or traversing the entire width of the membrane are globular proteins. Hydrophobic and electrostatic molecular interactions, along with the cytoskeleton, maintain the association of components, regionalization, and structural integrity of the membrane. Throughout the fish, and even within each cell, membranes may possess different morphological and biochemical attributes (Crockett and Hazel, 1995; Schulthess and Hauser, 1995). External influences such as diet and temperature may also alter membrane composition and character (Crockett and Hazel, 1995). Specific membranes may contribute to the characteristic disposition of xenobiotics according to their particular properties. All membranes, however, exhibit a basic structural similarity, which provides an operative model for their interaction with xenobiotic chemicals.

Diffusion

A primary pathway for xenobiotic transport across lipid membranes is by passive diffusion. Moderately nonpolar, lipid-soluble compounds diffuse easily across biological membranes. Polar and very nonpolar compounds diffuse across membranes less easily, although for different reasons. Polar compounds have limited access to the nonpolar portion of membranes, due to their low lipid solubility. In contrast, evidence suggests that very nonpolar compounds may be sequestered by membranes, limiting their movement. The net result of these interactions is that chemical transport from the external environment into plasma, interstitial fluid, intracellular fluid, and even into cell organelles is dictated by the ability of the compound to diffuse across membranes.

In general, the rate of diffusion across a membrane is directly proportional to the membrane–water partition coefficient of a chemical (assuming that the membrane is bathed on both sides by an aqueous medium), as well as its diffusion coefficient (for diffusion within the membrane) and the concentration gradient (Hunn and Allen, 1974; Spacie and Hamelink, 1982). The diffusion coefficient is a function of molecular size, conformation, and the presence or absence of particular functional groups. Specific to each membrane is a molecular weight or size discrimination profile, and the passive diffusion of large molecules may be prevented altogether. The membrane–water partition coefficient is a measure of the

relative solubility of the compound in the membrane and water. This coefficient may be difficult to measure. Lipid–water partitioning is often used, therefore, as a surrogate for membrane–water partitioning. The relationship of these factors can be summarized using Fick's law of diffusion:

$$\text{Flux} = \left(DP_{mw}/h\right)\left(C_1 - C_2\right) \tag{3.1}$$

where D is the diffusion coefficient (cm^2/sec), P_{mw} is the membrane–water partition coefficient (unitless), h is the thickness of the diffusion path or membrane (cm), and C_1 and C_2 are toxicant concentrations on each side of the membrane (mg/cm^3). When collected together, D, P_{mw}, and h define a permeability coefficient with units of cm/sec. For a unit area membrane, the rate of toxicant transfer across the membrane (flux) has units of mg/sec/cm^2. Multiplication by the membrane surface area provides a measure of total flux with units of mg/sec.

Flux that is directly proportional to the concentration gradient ($[C_1 - C_2]/h$) is first order and linear and progresses by a fractional rate constant. Numerous factors may influence the concentration of chemical available for diffusion, including external and internal pH partitioning phenomena, binding to organic material in water, and plasma protein binding. Composite fractional rate constants take these features into account for a given compound.

Empirical evidence suggests that non-ionized compounds diffuse more readily across membranes than ionized forms. These observations have important implications for membrane flux of weak organic acids and bases. If the non-ionized moiety has a lipid–water partition coefficient that favors membrane penetration, it will tend to reach an equilibrium concentration on both sides of the membrane, while the ionized form may be much more limited in its movements. As an approximation, therefore, the equilibrium concentration across the membrane will be based on the concentration of the non-ionized or lipophilic form, with Fick's law of diffusion applying only to this non-ionized form. The ratio of the two ionization states is dependent on the dissociation constant (pK_a) of the compound and the pH of the surrounding media. This relationship is described by the Henderson–Hasselbach equation:

$$\textit{For weak organic acids:}\ \text{pH} = pK_a + \log\ (\text{ionized/non-ionized}) \tag{3.2}$$

$$\textit{For weak organic bases:}\ \text{pH} = pK_a + \log\ (\text{non-ionized/ionized}) \tag{3.3}$$

For a weak organic acid, a decrease of one pH unit results in a tenfold increase in the concentration of the non-ionized form. Conversely, an increase of one pH unit results in a tenfold increase in the concentration of the ionized form. Weak organic bases behave in the opposite manner; for example, a weak organic acid (pK_a of 7) in moderately acidic water (pH of 5) would exist in a non-ionized/ionized ratio of 100/1. For a weak organic base, the ratio would be 1/100. The effect of pH on the non-ionized/ionized ratio can be extreme; thus, a pH change from 5 to 1 would change the non-ionized/ionized ratio of a pK_a 7 organic acid from 100/1 to 1,000,000/1.

Differences in pH across biological membranes will cause the degree of ionization to differ on either side of the membrane. The side with the greatest degree of ionization may have a much higher total xenobiotic concentration because the equilibrium distribution of chemical is based on concentrations of the diffusing non-ionized form (Figure 3.2). This phenomenon is referred to as *ion trapping*. The theoretical equilibrium concentration ratio (R_{xy}) of a xenobiotic across a membrane with two sides, x and y, can be calculated as follows:

$$\textit{For weak organic acids :}\ R_{xy} = \frac{1 + \text{anti}\log\left(pH_x - pK_a\right)}{1 + \text{anti}\log\left(pH_y - pK_a\right)} \tag{3.4}$$

$$\textit{For weak organic bases :}\ R_{xy} = \frac{1 + \text{anti}\log\left(pK_a - pH_x\right)}{1 + \text{anti}\log\left(pK_a - pH_y\right)} \tag{3.5}$$

With respect to these relationships, fish present a number of interesting nuances that are not typically encountered in mammals. While ion trapping in mammals is generally limited to internal membranes

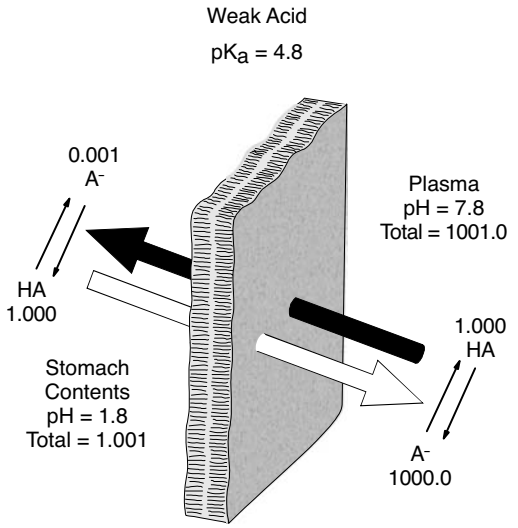

FIGURE 3.2 Distribution of a weak organic acid (pK_a = 4.8) on either side of a membrane separating the plasma and stomach contents, illustrating the concept of ion trapping as a result of pH differences. HA is the non-ionized form of a xenobiotic which can easily diffuse across the membrane; A^- is the ionized form with restricted diffusion. Numerical values represent the relative abundance of each form predicted by the Henderson–Hasselbach equation using compartment pH and the xenobiotic pK_a.

(e.g., blood to gastrointestinal tract, blood to milk, blood to urine), ion trapping in fish may occur across external surfaces such as the gills and skin (blood to water). The range of environmental pH values that will support fish populations is about 5 to 10. For a given compound, therefore, chemical flux across the gills and skin may occur rapidly or very slowly, depending on the ambient pH. Physiological differences between fish and mammals also exist. Most fish species have blood pH values that range from 7.7 to 8.0. For most mammals, the blood pH is approximately 7.4. In addition, nearly 15% of all fish species do not possess an acid-secreting stomach (Grondel et al., 1987). Finally, the pK_a for a particular compound or functional group will vary somewhat with temperature. Because fish are poikilotherms, changes in chemical dissociation may occur as they encounter different temperature environments.

Specialized Membrane Transport

Although simple diffusion controls many aspects of xenobiotic disposition, there are, in addition, specialized membrane transport systems that control chemical movements in specific cells and tissues. The specificity of these systems may be broadly based or extremely narrow. An example of the former is provided by systems that generically transport charged molecules, while the latter would include transporters responsible for nutrient and mineral uptake. Many of these carrier-mediated processes may contribute to membrane transport of xenobiotic compounds.

Compounds that are lipophilic and remain so throughout their residence in the body tend to be retained for extended periods of time. Processes such as glomerular filtration, passive tubular diffusion, and biliary excretion contribute to their elimination; however, the amount of compound associated with the aqueous phase of tissues, blood, and excretory products may be a small fraction of the total contained within the animal. Biotransformation accelerates the elimination of many lipophilic compounds by the converting them to more polar metabolites. Some of these metabolites retain enough lipid solubility to diffuse across membranes while exhibiting enough polarity to partition into aqueous phases. Other metabolites, including conjugated products of phase II metabolic pathways, are highly polar and do not diffuse as readily as theh parent compound across biological membranes. Membrane transporters play an important role in the elimination of these compounds.

Because the total number of enzyme molecules and membrane transporters contained within a tissue is finite, the kinetics of these systems is often saturable or capacity limited. The result is that somewhere

along the substrate concentration continuum competition for reaction sites results in a maximal rate of metabolism or transport. Under these conditions, the rate of metabolism or transport becomes independent of substrate concentration and is said to exhibit zero-order kinetics. Saturation of capacity-limited systems can result in profound effects on xenobiotic distribution and disposition.

Carrier-Mediated Transport

Carrier-mediated membrane transport occurs by rapidly reversible interactions between components of the membrane and the transported substance. These carriers generally exhibit selectivity for chemicals that possess specific characteristics; however, multiple chemicals possessing these characteristics may compete for the carrier. Saturability and competitive inhibition are characteristic of carrier-mediated transport. Active transport is a transmembrane carrier-mediated process that requires the direct expenditure of energy. The biological energy source may exist in several forms, including the nucleoside adenosine triphosphate (ATP) or as a transmembrane ion gradient. Of the latter, sodium gradients are most common. Active transport mechanisms are widely found in aquatic species and support a variety of physiological processes; for example, amino acids and carbohydrates are actively absorbed in the intestine of fish using a coupled sodium gradient (Ash, 1985; Jobling, 1995). Documentation of xenobiotic transport by such systems is increasing. Transporters with structural and functional similarities to multidrug resistance-associated proteins (Mrps), P-glycoprotein (Pgp), and organic anion transporter polypeptides (Oatps) have been identified in fish (Ballatori et al., 1999; Curtis et al., 2000; Doi et al., 2001; Hemmer et al., 1995; Kleinow et al., 2000; Masereeuw et al., 1999, 2000; Miller, 1995; Rebbeor et al., 2000). These carriers may transport xenobiotics in the canaliculi of the liver, the mucosa of the intestine, and the proximal tubule of the kidney. Additional carrier proteins have been associated with the movement of metals in fish such as silver across the gill (Bury et al., 1999) and iron transport across the yolk sac to the circulation (Donovan et al., 2000). Facilitated diffusion is a carrier-mediated process that does not require energy. Movement of the transported compound may occur in either direction across the membrane but the net movement follows the downhill electrochemical gradient much like passive diffusion. Unlike passive diffusion, however, facilitated diffusion may be capacity limited because the number of carriers is limited.

Pinocytosis

Pinocytosis is a process whereby the cell membrane invaginates, capturing external material within the lumen of a vesicle. The vesicle may empty its contents within the cell or fuse with another portion of the cell membrane, releasing its contents to the intercellular space. Uptake of macromolecular proteins by pinocytosis is well documented in the fish intestine (Rombout et al., 1985; Stroband and Kroon, 1981; Stroband et al., 1979). It is likely that transport of particulate or proteinaceous toxicants in fish occurs by this process, but verification is required.

Paracellular Pathway

Evidence in mammals suggests that tight junctions between neighboring cells are permeable to water, inorganic cations, and to a limited extent xenobiotics (Hoensch and Schwenk, 1984). The degree to which xenobiotics move by diffusion along these pathways is currently unknown for fishes.

Absorption, Distribution, and Excretion

Xenobiotic Absorption

Xenobiotics are absorbed by fish across the gills, skin, and gut. In terms of gross morphology, these structures differ greatly from one another. All three, however, possess two basic features that contribute to their role as chemical exchange surfaces: (1) large surface area, and (2) separation of the environment, or extension thereof, from the circulatory system by a membrane consisting of one, two, or a few cell layers. The path that a chemical takes when it is absorbed at one of these surfaces can be generally

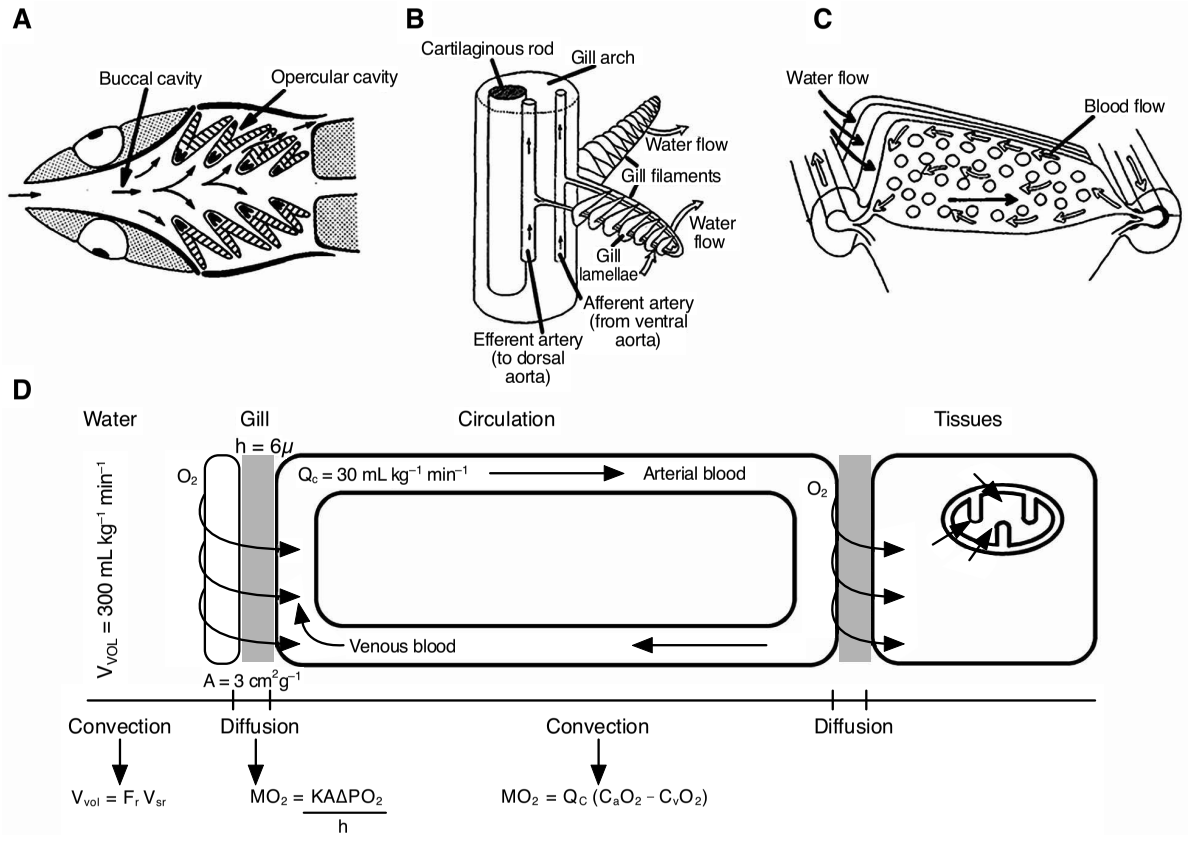

FIGURE 3.3 Anatomic and physiological model for oxygen uptake at fish gills. (A) Section through the head of a generalized teleost showing four pairs of gill arches; arrows show the direction of water flow through the buccal and opercular cavities. (B) Part of a gill arch showing two gill filaments with rows of lamellae on the ventral and dorsal surface. (Adapted from Wedemeyer, G.A. et al., *Environmental Stress and Fish Diseases*, TFH Publications, Neptune City, NJ, 1976.) (C) Transverse section of a gill filament showing a lamellae in side view; circles represent pillar cells. (D) Quantitative model for gas transfer in fish: V_{vol}, ventilation rate; F_r, ventilation rate; V_{sr}, respiratory stroke volume; MO_2, oxygen consumption rate; K, Krogh's permeation coefficient of diffusion; A, functional gill surface area; ΔPO_2, mean water-to-blood oxygen partial pressure gradient; h, diffusion distance; Q_c, cardiac output; C_aO_2, arterial blood oxygen content; C_vO_2, venous blood oxygen content. (Adapted from Perry, S.F. and McDonald, G., in *The Physiology of Fishes*, Evans, D.H., Ed., CRC Press, Boca Raton, FL, 1993, pp. 251–278.)

described as follows: (1) presentation to the absorbing epithelium in water or gut contents; (2) transport across the epithelium into blood; (3) incorporation into blood, including binding to plasma proteins; (4) transport via the systemic circulation of freely dissolved and plasma-bound chemical to various tissues; and (5) transport from blood into tissues. The character of the external medium may substantially determine which forms of a compound are presented to these exchange surfaces, thereby influencing the overall rate of uptake. The external medium may also impact uptake through its effects on the structure and function of the membrane itself. From mass-balance considerations, the rate of uptake cannot exceed the rate at which chemical is presented to the exchange surface. Rates of diffusion across the membrane barrier and removal by the circulatory system also have the potential to limit the overall rate of chemical uptake.

The Gills

Branchial Structure and Function

The anatomy of fish gills reflects their primary function as a gas-exchange, osmoregulatory, and excretory organ (Figure 3.3). Gill ventilation is accomplished using a two-phase pump system (Figure 3.3A). The first phase involves suction generated by opening the opercular flaps that cover the posterior portion of the branchial cavity. This draws water through the mouth into the buccal cavity. The second phase utilizes pressure, generated by the contraction of muscles lining the buccal cavity, to force water from the buccal cavity through the gill arches into the opercular cavity. During the pressure phase of the respiratory cycle, the oral valve prohibits water flow out of the mouth and directs water over the respiratory surface.

The respiratory surface, where branchial uptake of oxygen occurs, is composed of eight gill arches, four on each side of the branchial cavity. Each gill arch has two rows of gill filaments, and each filament is covered, top and bottom, with gas-exchange units called *secondary lamellae* (Figure 3.3B). The lamellae are thin-walled, sac-like structures composed of two epithelial cell layers held together by numerous pillar cells (Figure 3.3C). These pillar cells create a blood space between the two epithelial layers. Oxygen-depleted venous blood flows through the lamellae, while oxygen-rich inspired water flows between the lamellae. In most species, blood and water flow in opposite directions, creating an efficient counter-current system for gas exchange. Oxygen that diffuses across the lamellar epithelium binds to hemoglobin and is transported by blood to the tissues where it is utilized. Diffusion of oxygen from the blood into tissues occurs at a rate dependent on the concentration gradient between the blood and individual cells of the tissues. A general model for gas transfer in teleosts is presented in Figure 3.3D.

Emphasis in this chapter is placed on those aspects of normal gill function that control branchial uptake of xenobiotic compounds. For further information on respiratory function in teleosts, the reader is referred to pertinent reviews (Perry and McDonald, 1993; Piiper and Scheid, 1984; Randall and Daxboeck, 1984). For detailed discussions of gill anatomy, see Hughes (1984) and Laurent (1984). Additional reviews cover osmotic, ionic, and acid–base regulation (Evans, 1993; Heisler, 1993; McDonald, 1983) and excretory function (Wood, 1993).

Branchial Absorption of Xenobiotics

The anatomical and physiological features of fish gills that promote efficient exchange of respiratory gases also contribute to uptake of xenobiotic compounds directly from water—namely, a thin membrane separating blood and water, large surface area, and high rates of counter-current blood (perfusion) and water (ventilation) flow. Direct measurements of chemical uptake across fish gills have been obtained using fish respirometer-metabolism chambers, which separate inspired and expired water flows (McKim and Goeden, 1982; McKim and Heath, 1983). Using this model system, McKim et al. (1985) measured branchial uptake rates in adult rainbow trout (*Oncorhynchus mykiss*) for a heterogeneous group of organic chemicals. These measurements suggested a consistent relationship between the uptake rate of a chemical and its relative hydrophobicity, as indicated by the log of its octanol–water partition coefficient (log K_{ow}; Figure 3.4A). Rate constants measured in subsequent studies with rainbow trout (Bradbury et al., 1986; McKim et al., 1986, 1987a,b) further substantiated this relationship. Uptake rates were low for chemicals with log K_{ow} values less than 1, increased about fourfold between log K_{ow} 1 and 3, leveled off between log K_{ow} 3 and 6, and declined when log K_{ow} exceeded 6. Working with a series of phenols, anisoles, and

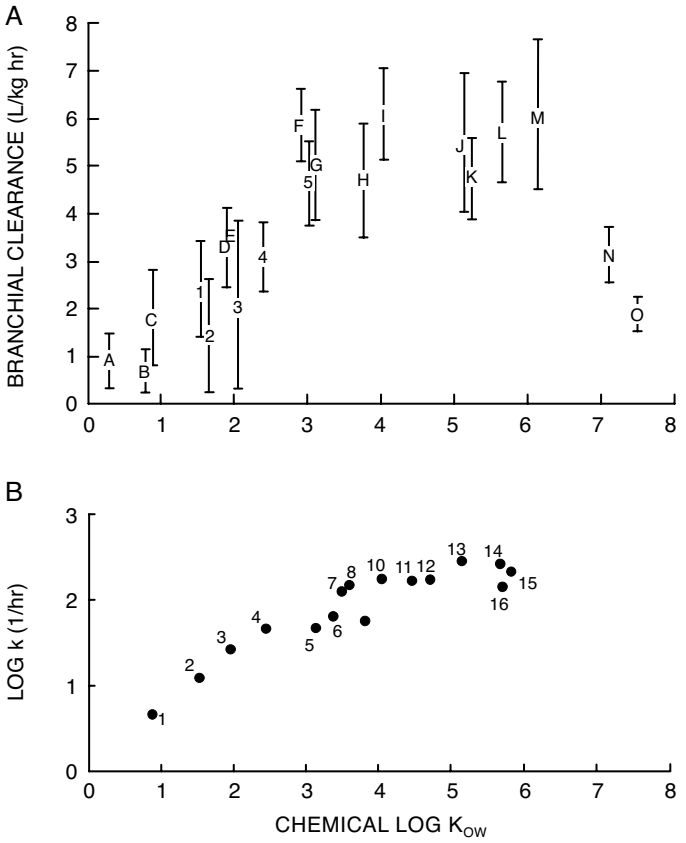

FIGURE 3.4 Relationship between the branchial uptake of chemicals by rainbow trout and guppies, and chemical hydrophobicity expressed as the log of the octanol–water partitioning coefficient. The uptake rate for rainbow trout is expressed as a clearance constant; that for guppies is given as the log of the uptake rate constant from water when pH < chemical pK_a. (A) *Rainbow trout sublethal exposures:* A, ethyl formate; B, ethyl acetate; C, 1-butanol; D, nitrobenzene; E, *p*-cresol; F, chlorobenzene; G, 2,4-dichlorophenol; H, 2,4,5-trichlorophenol; I, 1-decanol; J, 1-dodecanol; K, pentachlorophenol; L, hexachlorobenzene; M, 2,2′,5,5′-tetrachlorobiphenyl; N, dinitrophenol; O, mirex. *Rainbow trout lethal exposures:* 1, benzaldehyde; 2, 2,4-dinitrophenol; 3, MS-222; 4, malathion; 5, 1-octanol. (Data from Erickson and McKim, 1990a.) (B) *Guppy sublethal exposures:* 1, butyric acid; 2, phenol; 3, benzoic acid; 4, 4-phenylbutyric acid; 5, 2,4-dichlorophenol; 6, 2-sec butyl-4,6-dinitrophenol; 7, 3,4,-dichlorobenzoic acid; 8, 2,6-dibromo-4-nitrophenol; 9, 2,4,5-trichlorophenol; 10, 2,4,6-trichlorophenol; 11, 2,3,4,6-tetrachlorophenol; 12, tetrachloroverathrol; 13, pentachlorophenol; 14, pentachloroanisol; 15, 2,4,6-trichloro-5-phenylphenol; 16, DDT. (Adapted from Saarikoski, J. et al., *Ecotoxicol. Environ. Saf.*, 11, 158–173, 1986.)

carboxylic acids, Saarikoski et al. (1986) found a similar relationship between absorption rate and log K_{ow} in the guppy (*Poecilia reticulata*) (Figure 3.4B). Additional studies with a homologous series of chlorinated ethanes in the channel catfish (*Ictalurus punctatus*) (McKim et al., 1994), fathead minnow (*Pimephales promelas*) (Lien et al., 1994), and lake trout (*Salvelinus namaycush*) (McKim et al., 1999a) have extended these observations to other species.

Several physiological and chemical factors may account for these trends in gill uptake with chemical hydrophobicity. Gobas et al. (1986) proposed that branchial uptake was regulated by chemical partitioning into lipid phases and diffusion through lipid membranes and associated aqueous layers. Both McKim et al. (1985) and Saarikoski et al. (1986) attributed increases in absorption between log K_{ow} 1 and 3 to greater membrane permeability for the more lipophilic compounds. Opperhuizen et al. (1985) suggested that gill membranes inhibit the uptake of lipophilic compounds that exceed a critical molecular volume. Other researchers, noting a downward trend in bioconcentration factors (BCFs; defined as the ratio of the chemical concentration in the fish to that in water) for compounds with log K_{ow} values greater than

6, proposed that structural attributes of these compounds limit branchial uptake (Bruggeman et al., 1984; Konemann and Van Leeuwen, 1980; Sugiura et al., 1978; Tulp and Hutzinger, 1978; Zitko, 1974; Zitko and Hutzinger, 1978). Alternatively, because uptake of these compounds is extremely slow, steady-state concentrations may not have been achieved in these studies, leaving the false impression of a downward deflection in BCF. Branchial uptake of highly lipophilic compounds may also be limited by binding to dissolved organic matter in the water, thereby reducing the concentration of the free or bioavailable form of the compound. Ionized organic compounds exhibit limited ability to cross the gill membranes and must be dealt with as a special group.

Water and blood flows through the gills maintain xenobiotic diffusion gradients across the gill epithelium. Norstrom et al. (1976), Neely (1979), and Bruggeman et al. (1981) suggested that branchial uptake may be correlated to the rate of water flow across the gills and thus to respiration rate. Gobas and MacKay (1987) considered multiple diffusion and flow steps to be part of the branchial exchange process. Barber et al. (1988) presented a hydrodynamic-based model for chemical bioconcentration in fish that incorporates effects of advection and diffusion in water flowing through the gills. Hayton and Barron (1990) suggested that blood flow, water flow, and diffusional barriers all have the potential to influence branchial flux, and they proposed a model based on the concept of serial resistance. Erickson and McKim (1990a,b) developed a counter-current model of branchial flux for non-ionized compounds that includes both flow and diffusion limitations, as well as chemical binding to dissolved organic material. Detailed descriptions of both models are given later in this chapter.

Physiological Impacts on Branchial Absorption

Gill ventilation and blood perfusion rates vary with activity level, temperature, and environmental oxygen tension (Barron et al., 1987a; Hughes, 1984; Randall and Daxboeck, 1984). Differences in environmental conditions and activity level also impact the toxicity and bioconcentration of chemicals in fish. We now understand that these observations are linked; changes in environment and activity increase or decrease branchial absorption of xenobiotics by impacting the rate-limiting processes that control this uptake.

Dissolved Oxygen—Four decades ago, Lloyd (1961) observed increases in the toxicity of several chemicals to rainbow trout as ambient oxygen concentrations were reduced from 100% to 30% of saturation. He suggested that the increase in toxicity was caused by an increase in ventilation volume that occurred at reduced oxygen concentrations, resulting in a larger quantity of chemical being brought in close contact with the gills. Lloyd's (1961) observations were supported by those of McKim and Goeden (1982), who measured changes in branchial uptake of endrin (log K_{ow} 5.02) in response to a stepwise decrease in afferent water oxygen concentration. Initial oxygen and endrin extraction efficiencies were nearly constant at oxygen saturation values of 100% and 80% but dropped progressively at oxygen saturation values of 50% and 30% (Figure 3.5A). Apparently, endrin extraction efficiency was maintained in the face of moderate increases in ventilation volume because diffusion was fast enough to clear lamellar channels of most of the chemical (i.e., there was no change in physiological dead space; see Hughes, 1984), or because the number of blood-perfused gill lamellae increased (Randall and Daxboeck, 1984). Observed declines in extraction efficiency may have been due to an increase in ventilatory stroke volume, resulting in greater bypass of water around the lamellar channels (i.e., increased anatomical deadspace), or an increase in physiological deadspace associated with increased water velocities. Because ventilation volume increased more than extraction efficiency decreased, total endrin uptake rate at 50% of oxygen saturation was about double that at 100% (Figure 3.5B). The additional decrease in oxygen saturation from 50% to 30% did not result in higher endrin uptake rates.

Opperhuizen and Schrap (1987) examined the impact of aqueous oxygen concentration on uptake of two polychlorinated biphenyl (PCB) congeners by guppies. Over the range of oxygen concentrations tested (60%, 40%, and 25% of saturation) they saw no differences in uptake of either chemical and suggested that a drop in chemical extraction efficiency occurred as ventilation volume increased in response to lower oxygen saturations. Based on these observations, the authors concluded that diffusional constraints have more impact on total chemical flux at the gill than changes in water flow. The results of McKim and Goeden (1982) suggest that this conclusion does not apply at higher oxygen concentrations (>60% of saturation), where uptake appears to be related to ventilation volume, at least for some fish.

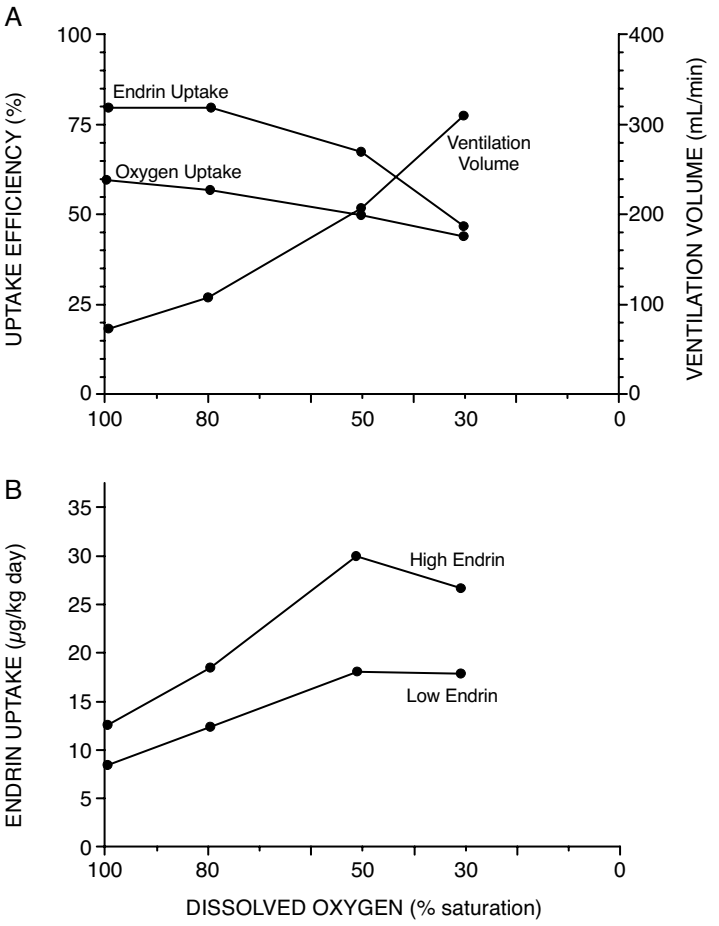

FIGURE 3.5 Effect of dissolved oxygen concentration on branchial uptake of endrin by brook trout. (A) Changes in ventilation volume, oxygen uptake efficiency, and endrin uptake efficiency. (B) Changes in endrin uptake rate at high (0.072 mg/L) and low (0.046 mg/L) waterborne concentrations. (Adapted from McKim, J.M. and Goeden, H.M., *Comp. Biochem. Physiol*., 72C, 65–74., 1982.)

Complicating these comparisons, however, is the issue of fish size. For small fish, such as the guppy, dermal absorption may account for up to 50% of total uptake of waterborne chemicals with high log K_{ow} values (Lien and McKim, 1993). Assuming that skin perfusion as a fraction of cardiac output remains constant, chemical absorption across the skin would change very little with a decrease in the oxygen content of water, reducing the overall effect of any change in oxygen content on total chemical uptake.

Temperature—Environmental temperature changes can cause dramatic changes in the metabolic rates of poikilothermic animals (Prosser, 1973), which affects their demand for oxygen. An increase in oxygen demand due to increased temperature is especially problematic because the solubility of oxygen in water is inversely related to temperature. To obtain more or less oxygen, a fish in most cases adjusts its ventilation volume and the number of perfused lamellae in contact with respiratory water (Randall, 1982). This suggests that, if chemical uptake was controlled by water flow across the gills, an increase in temperature would increase branchial uptake rate in a manner qualitatively similar to that of a decrease in oxygen content. Changes in temperature also affect cardiac output and could potentially impact branchial absorption if blood flow to the gills was a limiting factor. Several authors have investigated the relationship between chemical uptake and oxygen consumption at different water temperatures. Using rainbow trout, Rodgers and Beamish (1981) reported that branchial uptake of methylmercury was

positively correlated with that of oxygen at two temperatures (10 and 20°C) and two levels of forced swimming. There was no indication of change in uptake efficiency for either methylmercury or oxygen. Similarly, Murphy and Murphy (1971) demonstrated a positive correlation between oxygen consumption and uptake of waterborne dichlorodiphenyltrichloroethane (DDT) by the mosquito fish (*Gambusia affinis*) at 5 and 20°C. The ratio of DDT uptake rate to fish weight was proportional to the ratio of oxygen consumption to fish weight at each of the two temperatures. The authors concluded that the uptake of DDT across the gills was influenced by changes in respiratory volume. Black et al. (1991) found that the branchial uptake of three moderately hydrophobic compounds—benzo(*a*)pyrene, 2,2′,5,5′-tetrachlorobiphenyl, and naphthalene—changed in direct proportion to oxygen consumption when rainbow trout were subjected to an acute reduction in temperature from 17°C to 8°C (Figure 3.6A). The decrease in oxygen consumption was accompanied by reductions in ventilation volume and ventilation rate (Figure 3.6B). Finally, it is well known that chemical diffusion rates in solution increase with temperature. Similar changes are expected to occur in biological membranes and would be important if diffusion across the gill epithelium limited the rate of branchial flux. Fish may also respond to prolonged changes in temperature by altering the molecular structure of the gill epithelium, changing its permeability to xenobiotic compounds.

The Gastrointestinal Tract

Gastrointestinal Tract Structure and Function

The gastrointestinal tract (GIT) of fishes functions in digestion, nutrient absorption, and excretion and as a barrier to the external environment. For some groups of fishes, the GIT also plays a role in osmoregulation, buoyancy, ion regulation, and placental nutrition. Diverse structural adaptations in different fish species reflect a wide array of digestive strategies. Gross differences among species include the presence or absence of an acid-secreting stomach, presence or absence of a gizzard, large differences in GIT length, and an absence or varying number of blind diverticula called *pyloric ceca* that project from the intestine near the pylorus of the stomach. Detailed reviews of fish GIT structure and function are provided elsewhere (Kapoor et al., 1975; Kleinow and James, 2001; Smith, 1989).

Common to most species, the buccal cavity is followed by a short and distensible esophagus; some type of stomach; proximal, middle, and distal intestine; and finally a rectal region. Along its length, the GIT changes histologically. Major features of the esophagus include numerous goblet cells, which provide lubrication, and primary, secondary, and tertiary folds, which allow distension during the ingestion and swallowing of food. A true stomach, when present, often contains cardiac glands that extend between the lamina propria and columnar epithelial cells lining the stomach lumen.

The structure of the intestine, including its diameter and the abundance of goblet cells and longitudinal folds, varies along its length. Villi and crypt regions, as classically defined in the mammalian intestine, are not evident in many fishes but are replaced by the longitudinal folds which exhibit varying degrees of branching (Field et al., 1978; Kapoor et al., 1975). As with mammals, the intestinal lumen of most fishes is lined by a simple columnar epithelium that possesses a brush border. Pyloric ceca are lined with columnar absorptive cells and are histologically similar to the adjacent intestine.

The proximal intestine (including, when present, pyloric ceca) provides the greatest surface area for absorption due to the relative abundance and length of longitudinal folds. Comparisons among species suggest that total absorptive surface area varies inversely with diet quality (Horn, 1989). For a given species, intestinal length, mucosal weight, and total gut surface area may vary inversely with acclimation temperature (Lee and Cossins, 1988).

The absorptive surface of the intestine consists of several structural and functional layers. On the lumen margin, overlying the mucosal epithelium, is an unstirred layer consisting of water and a layer of mucus. The mucus is an organic-based permeable gel that is constantly renewed by goblet cells of the mucosa. The thickness of the mucus layer changes by region and is generally greatest in distal sections of the intestine. The composition of mucus may also differ among intestinal regions (Trevisan, 1979) and species (Jirge, 1970). A third, largely functional layer is interposed between the epithelial cell surface and the unstirred layer. Often referred to as the *acid microclimate*, this layer is a cell membrane charge barrier associated with the epithelial cells. The absorptive pathway extends through

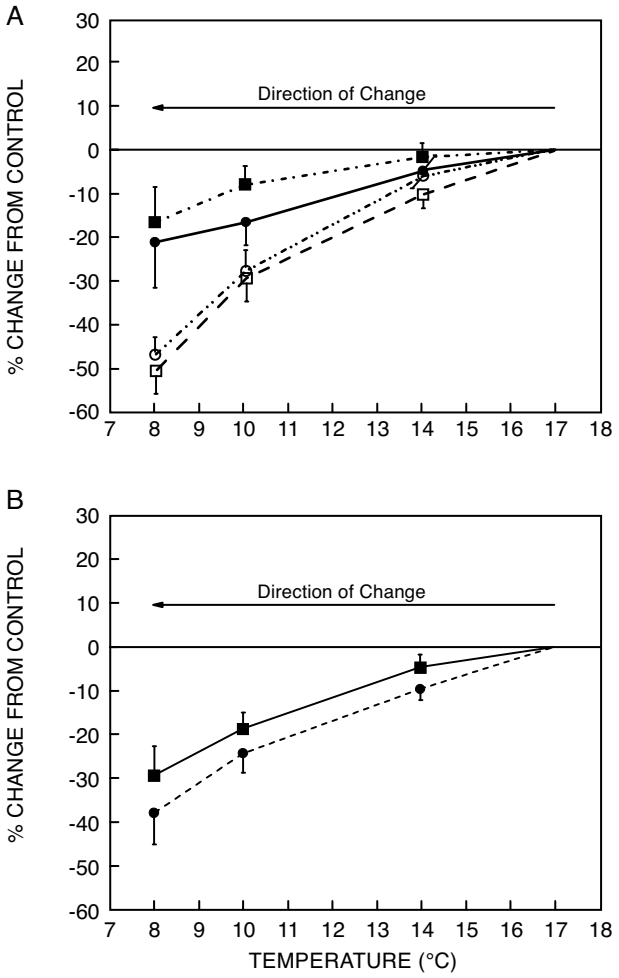

FIGURE 3.6 Effect of temperature on branchial uptake of benzo(*a*)pyrene, 2,2′,5,5′-tetrachlorobiphenyl, and naphthalene by rainbow trout. (A) Changes in chemical uptake efficiency (solid squares), oxygen uptake efficiency (solid circles), chemical uptake rate (open squares), and oxygen consumption rate (open circles). (B) Changes in ventilation rate (solid squares) and ventilation volume (solid circles). Each point represents the mean percentage change (±SE) from the control value measured at the acclimation temperature, calculated by pooling data for all three compounds. (Adapted from Black, M.C. et al., *Physiol. Zool.*, 64, 145–168, 1991.)

and beyond the epithelial cells to the lamina propria, where vascular elements of the intestinal tract are located. The lamina propria, besides providing a structural matrix supporting the mucosal epithelia, serves as a storage and staging area for vascular transport of nutrients and associated xenobiotics.

Along the length of the GIT are regional differences in luminal pH, fluid fluxes, transporter systems, enzymes, and enzymatic activities. Qualitatively, the digestive process in fish is similar to that of other vertebrates. Quantitative differences exist, however, especially in relation to digestive enzyme concentrations and activities. Regional differences in these parameters, combined with structural differences, restrict specific functions to more or less discrete locales. The regional nature of nutrient absorption is one outcome of these differences. The residence time of a food bolus in any given part of the GIT depends on many physical and physiological factors. In general, nutrient uptake and blood flows are highly regionalized and respond to local stimuli initiated by the food bolus. As the food bolus moves through the GIT, the character of the ingesta changes, as does the volume. Nutrients are absorbed and mucus, bacteria, and epithelial cells are added. As a result, different regions of the absorptive surface are presented with ingesta that differs in composition.

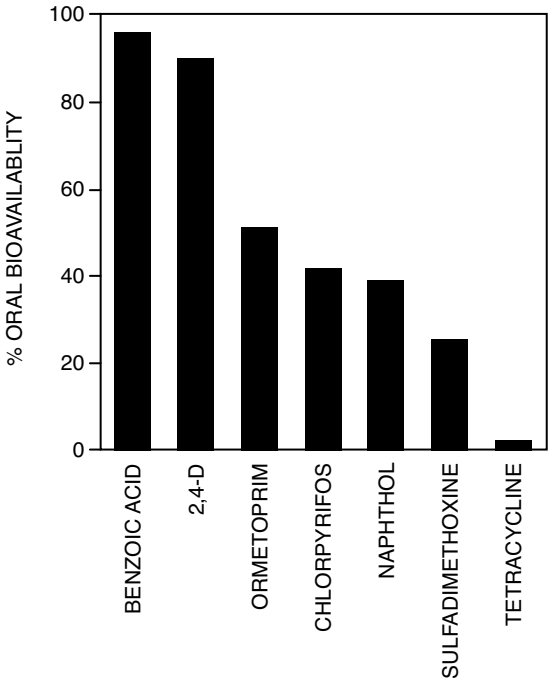

FIGURE 3.7 Oral bioavailability of seven chemicals in channel catfish, expressed as a percentage of the administered dose. (Data are from Barron et al., 1991; Michel et al., 1990; Plakas and James, 1990; Plakas et al., 1990, 1992b; Stehly and Plakas, 1992.)

Gastrointestinal Absorption of Xenobiotics

The absorption of xenobiotics from the GIT depends on a multiplicity of events in four general spheres of interaction: (1) within the intestinal lumen, (2) at the absorptive surface, (3) within the enterocytes, and (4) at the enterocyte–vascular interface. Physical, chemical, and biochemical events within the intestinal lumen determine the chemical form and integrity of a compound and its availability for uptake across the gut epithelium. Qualitative and quantitative characteristics of the diet play an especially important role in this environment. On the apical surface of the enterocytes, physical and chemical properties of the membrane and associated unstirred layer, as well as the transporter complement within the membrane, significantly influence xenobiotic uptake. Within the enterocytes, biotransformation enzymes, carrier molecules, and cellular components further impact xenobiotic concentration and movement. Transport across the enterocyte basolateral membrane into blood is dependent on factors similar to those operating at the apical membrane. The composition and flow rate of blood may also influence uptake. Directly impacting all of these processes is the character of the xenobiotic and fish species under consideration. The relative amount of an ingested compound that reaches the systemic circulation is defined as *dietary* or *oral bioavailability.* Oral bioavailability may be expressed relative to 100% (complete bioavailability) using the area under the blood concentration–time curve (AUC), referenced to that of an intravascular dose (Gibaldi and Perrier, 1982). Oral bioavailability in fish is species and chemical specific and varies widely (Figure 3.7).

Dietary uptake also may be characterized by measuring chemical residues in tissues after feeding fish a defined ration of contaminated food. The uptake efficiency calculated in this manner is termed *dietary assimilation efficiency*, and it reflects the net result of dietary uptake and subsequent elimination (Penry, 1998). A third means of characterizing dietary uptake is to estimate the efficiency with which ingested compounds are absorbed across the GIT. Typically, this is done by fitting the whole-fish chemical concentration data to a derivative form of the mass-balance equation that describes dietary uptake and elimination (Bruggeman et al., 1984). This approach yields a dietary absorption efficiency constant, the

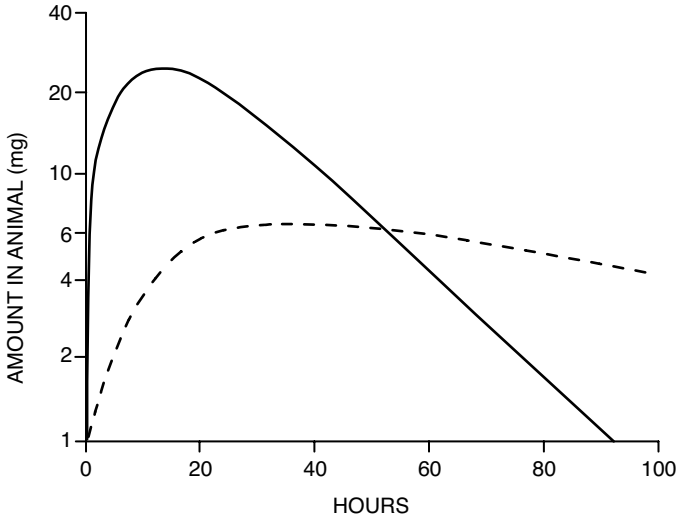

FIGURE 3.8 Simulated effect of gut absorption rate on whole-animal chemical kinetics. Simulation conditions: one-compartment open model; dose, 50 mg; elimination half-life, 20 hours; absorption half-life, 7 hours (solid line) or 70 hours (dashed line).

calculation of which mathematically factors for the effect of chemical elimination, including biotransformation. Dietary absorption efficiencies have been reported for a number of halogenated organic chemicals in several fish species (Burreau et al., 1997; Gobas et al., 1988; Muir and Yarechewski, 1988; Niimi and Oliver, 1988; Opperhuizen and Sijm, 1990). Gobas et al. (1988) compiled much of this information and related measured absorption efficiencies to chemical log K_{ow} (Figure 3.9). At log K_{ow} values less than 6, reported values range from 30 to 70%, averaging about 50%. A log K_{ow}-dependent decrease in absorption efficiency is apparent for compounds with log K_{ow} values greater than 7. When a compound is administered as a single dietary dose, the rate of absorption influences chemical concentrations achieved in tissues. At a fixed rate of elimination, an increase in the rate of absorption results in relatively higher maximum concentrations. Decreasing the rate of absorption results in relatively lower maximum concentrations that tend to be maintained for longer periods of time (Figure 3.8). When multiple doses of a compound are administered in the diet, the rate of absorption does not generally impact steady-state concentrations.

Bengtsson et al. (1979) fed bleaks (*Alburnus alburnus*) a technical mixture of chlorinated paraffins (CPs) and found that dietary uptake was inversely related to carbon-chain length and the extent of chemical chlorination. In a subsequent study, dietary uptake and accumulation of CPs by rainbow trout was variably related to the extent of chlorine substitution, depending on carbon-chain length (Fisk et al., 1996). Chlorination of short-chain-length compounds appears to increase accumulation by reducing biotransformation. In contrast, high levels of chlorine substitution may limit the diffusion of CPs with longer chain lengths across the mucosal membrane and perhaps incorporation into micelles. In several fish species, dietary uptake of high-molecular-weight CPs (MW > 600) was low or nonexistent (Bengtsson et al., 1979; Lombardo et al., 1975; Zitko, 1974). In general, lipophilic contaminants are more readily taken up from the diet if they have a molecular weight less than 600 (Bruggeman et al., 1984; Niimi and Oliver, 1988), low extent of chlorine substitution (Tanabe et al., 1982), and small molecular volume (<0.25 nm^3) (Niimi and Oliver, 1988).

Dietary uptake of lipophilic contaminants may depend on the existing contaminant burden in the fish. Doi et al. (2000) showed that the uptake of [^{14}C]-3,3′,4,4′-tetrachlorobiphenyl ([^{14}C]-PCB 77) from *in situ* perfused catfish intestines was reduced by dietary pretreatment with unlabeled PCB 77. The cause of this decrease was unclear but did not appear to be related to changes in first-pass intestinal metabolism. *In vivo* studies with catfish have also demonstrated lower dietary uptake of PCBs following preexposure in the diet (Hansen et al., 1976) or exposure to contaminated sediments (Dabrowska et al., 1996). Kinetic

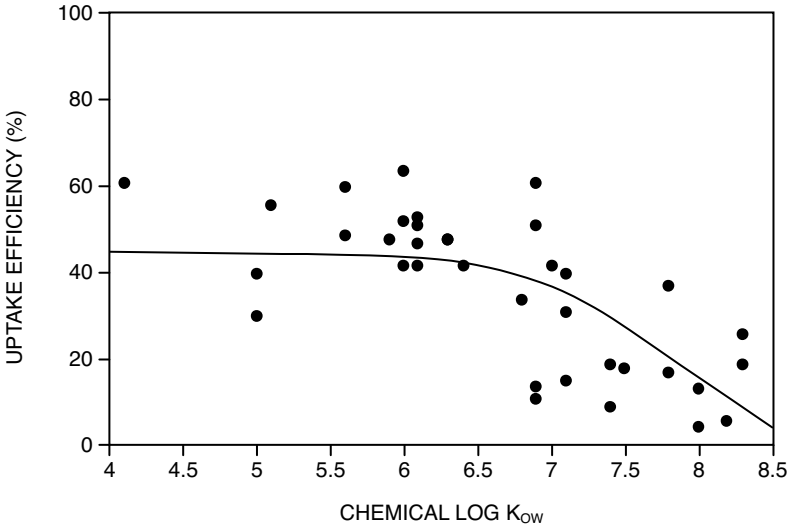

FIGURE 3.9 Dependence of dietary absorption efficiency on chemical log K_{ow}. Solid circles represent values summarized by Gobas et al. (1988). The solid line was generated using the fugacity model presented by Gobas et al. (1988).

models developed by Barber et al. (1991) and Clark et al. (1990) were used to simulate dietary uptake of PCBs by fish. Both models predicted that dietary uptake will decrease with increasing chemical body burden due to a decrease in the concentration gradient for uptake.

A few studies have examined the effect of dosage on the oral bioavailability of more water-soluble xenobiotics in fish. The bioavailability of the antibiotic oxolinic acid in rainbow trout was reported to be 14.3% (Cravedi et al., 1987), 13.6% (Bjorklund and Bylund, 1991), 38.1% (Cravedi et al., 1987), and 91% (Kleinow et al., 1994) after oral administration of the drug at 100, 75, 20, and 5 mg/kg, respectively. These results suggest that the bioavailability of oxolinic acid is higher at lower dosages. Other studies with Atlantic salmon (*Salmo salar*) indicated no difference in bioavailability when fish were administered oxolinic acid in feed at 9 and 26 mg/kg (Hustvedt et al., 1991). The bioavailability of sulfadimethoxine in rainbow trout decreased slightly when the dosage was increased threefold from 42 to 126 mg/kg (Kleinow et al., 1992).

Studies with oxolinic acid in yellowtail (*Seriola quinqueradiata*) showed that oral bioavailability was inversely related to particle size (Endo et al., 1987). The bioavailability of the sodium salt of sulfadimethoxine was nearly double that of the non-salt form (63 to 34%) (Kleinow et al., 1992). In each case, bioavailability appears to have been controlled in part by solubility considerations.

Physiological Impacts on Gastrointestinal Absorption

Relatively little information is available regarding the direct effects of GIT structure and function on dietary uptake of xenobiotics by fish. It is evident from nutritional studies, however, that pH gradients, gastrointestinal passage times, blood-flow patterns, and other aspects of digestive physiology vary with temperature and feeding frequency, as well as meal composition and size. Work with the mammalian GIT also indicates that these physiological features influence the absorption of xenobiotics.

Digestion—Approximately 15% of teleost fishes lack a true stomach (Grondel et al., 1987). Significant longitudinal pH gradients are not observed in these agastric species. The stomach pH of gastric species generally ranges from 1 to 4 (Bowen, 1981; Moriarty, 1973; Payne, 1978), and that of the intestine ranges from 6.5 to 9 (Fish, 1960; Nagase, 1964). Regional pH in the GIT may be an important determinant of absorption for some xenobiotics (Guarino et al., 1988). This would be especially true for weak organic acids and bases. In addition, pH can impact xenobiotic integrity, suggesting that differences in this regard may exist between gastric and agastric species.

Because of its location, high surface area, and role in nutrient uptake (Gauthier and Landis, 1972; Noaillac-Depeyre and Gas, 1976, 1979), the proximal intestine is the most important site for xenobiotic absorption in fish. It is also clear that other regions of the teleost intestine are capable of significant chemical uptake (Kleinow et al., 1992; Nichols et al., 2004a). Regionally selective absorption has been demonstrated with both organic chemicals and metals (Pentreath, 1976; Shears and Fletcher, 1983).

Many xenobiotics are absorbed concurrently or in conjunction with specific nutrient classes. Digestive processes liberate xenobiotics from the food matrix, transport them to the mucosal epithelium, and control their presentation to the absorptive surface. Gut uptake of lipophilic contaminants has been strongly associated with digestion and assimilation of dietary lipid. Mechanical mixing, pancreatic lipases, and bile salts disperse dietary lipid and contribute to the formation of an equilibrium phase consisting of small mixed micelles (Kleinow and James, 2001). Lipophilic contaminants reside in the interior of these micelles, while the hydrophilic exterior allows the micelles to remain soluble in the aqueous milieu of the luminal contents. Micelles provide a packaging and transport phase that can traverse the unstirred water layer of the mucosa. Upon reaching the enterocytes, the micelles are dissociated by the combined action of pH changes and mucosal lipases, liberating stored xenobiotic. This process creates a highly localized concentration gradient of both xenobiotic and fatty acids across the absorptive epithelium.

Changes in the character of the ingesta can facilitate or hinder the absorption of xenobiotics; for example, moderate levels of dietary lipid appear to facilitate the absorption of lipophilic contaminants, but low or very high lipid diets are associated with reduced uptake efficiency (Andrews et al., 1978; Van Veld, 1990). High levels of dietary fat may overwhelm the ability of the system to digest and remove this material. Unprocessed lipid would then provide a partitioning phase for lipophilic compounds within the gut lumen. In addition, qualitative features of dietary lipid such as fatty acid chain length and composition may determine xenobiotic solubility in micelles and subsequent systemic availability (Doi et al., 2000).

Dietary lipids absorbed by the enterocytes are exported as lipoproteins to interstitial spaces of the lamina propria and to the vasculature. Mammals release these products as chylomicrons and very-low-density lipoproteins. Fish appear instead to release very-low-density lipoproteins, high-density lipoproteins, and free fatty acids (Babin and Verneir, 1989; Iijima et al., 1985, 1990). Because fish do not possess a lymphatic system, the potential for xenobiotics in lymph to bypass the liver, as seen in mammals, does not exist. This feature may contribute to the hepatic extraction of some xenobiotics before they reach the systemic circulation.

Gastric Evacuation and Gut Transit Time—Considerable effort has been expended to characterize rates and patterns of gastric evacuation in fish. Exponential decay models have been successfully employed to describe gastric evacuation in several species (Brodeur and Pearcy, 1987; He and Wurtsbaugh, 1993; Persson, 1986; Ruggerone, 1989). This approach suggests that the gastric emptying rate at any time point is proportional to the amount of food remaining in the stomach. Generally, the rate constant for gastric emptying increases with acclimation temperature and decreases with food particle size (Jobling, 1987; Windell et al., 1976). Meal size has been reported to increase, decrease, and have no effect on gastric evacuation rates. These apparent differences may be due in part to the use of dry and wet weights to express stomach contents data (Elliott, 1991). Variable relationships also exist between dietary composition and gastric emptying; increased dietary lipid has been shown to delay gastric emptying in some cases (Windell et al., 1969) but not others (Persson, 1982).

Relatively little is known of total gut transit times in fish. When blennies (*Blennius pholis*) were fed a single meal of fresh lugworms, the time required for intestinal emptying ranged from 20 to 30 hours and tended to increase with meal size (Grove and Crawford, 1980). Gut transit times for a stomachless species, the bighead carp (*Aristichthys nobilis*), ranged from about 7 to 13 hours and decreased with increasing body size (Opuszynski and Shireman, 1991). The time required for rainbow trout to completely digest a meal of live fathead minnows was less than 48 hours; however, a small amount of material was retained within the intestines between feedings (Nichols et al., 2004a). This finding suggests that complete evacuation of one meal depends on the consumption of additional meals. Gut transit times tend to decrease with increased acclimation temperature (Hofer et al., 1982; Shrable et al., 1969).

In mammals, an increase in feeding rate generally results in decreased gut transit time and reduced digestibility. Both of these outcomes may contribute to reductions in xenobiotic bioavailability. Limited information suggests that similar considerations also apply to fish; for example, the oral bioavailability of methylmercury in catfish was found to be inversely related to meal size (McCloskey et al., 1998). In other studies, the bioavailability of oxolinic acid to catfish was found to increase with acclimation temperature (Kleinow et al., 1994), and that of enrofloxacin in rainbow trout decreased (Bowser et al., 1992). The apparent disagreement between these observations may be due to competing effects of temperature on gut transit time and digestibility. By increasing the transit time, a decrease in temperature might be expected to promote bioavailability; however, an accompanying decrease in digestibility might slow the release of chemical from the food matrix. The effect of temperature on bioavailability may depend, therefore, on which factors limit dietary uptake for a given compound, diet, and species. In some cases, chemical desorption from the food source may be the rate-limiting determinant of dietary uptake; for example, desorption half-lives for hydrophobic contaminants from ingested sediments may be greater than a week, which most likely exceeds the intestinal transit time of most organisms (Schrap, 1991).

Blood Flow—The GIT is supplied by a relatively complex vascular network (Farrell et al., 2001; Thorarensen et al., 1991). Blood flow to the GIT as a percentage of cardiac output in resting, unfed fish appears to vary considerably among species. Values based on the distribution of injected microspheres range from less than 1% in channel catfish (Schultz et al., 1999) to nearly 20% in rainbow trout (Barron et al., 1987a). Doppler flow probes were used to measure blood flow through the coeliaco-mesenteric arteries of Chinook salmon (Thorarensen et al., 1993), Atlantic cod (*Gadus morhua*) (Axelsson and Fritsche, 1991), and red Irish lord (*Hemilepidotus hemilepidotus*) (Axelsson et al., 2000). In each case, the measured flow rate accounted for 30 to 40% of total cardiac output, leading to the suggestion that microsphere methods underestimate blood flow to the GIT (Thorarensen et al., 1993). In addition to the GIT, however, the coeliaco-mesenteric artery provides blood to several internal organs including (depending on species) the liver, spleen, swim bladder, and gonads (Smith and Bell, 1975). Blood flow to the GIT increased substantially after feeding (Axelsson and Fritsche, 1991; Axelsson et al., 1989, 2000) and decreased during strenuous exercise (Axelsson and Fritsche, 1991). In mammals, the increase in blood flow that occurs with feeding tends to be localized to regions that contain food, and an increase in flow through the coeliac artery generally precedes a flow increase in the mesenteric arteries. Similar changes probably occur in fish (Axelsson et al., 2000). The effect of these changes in blood flow on dietary uptake of xenobiotic compounds is currently unknown. It may be speculated, however, that an increase in blood flow would promote uptake by maintaining an inward gradient for chemical diffusion.

The Skin

Skin Structure and Function

The skin of a fish functions as a physical barrier to separate the internal and external environments. As such, it maintains the ionic and osmotic integrity of the internal environment, provides protection from abrasion and disease, and participates in the exchange of respiratory gases. The skin is one of the largest organs of the fish body (approximately 10% of body weight) and may act as an important exchange surface for the absorption and excretion of xenobiotic chemicals.

Grossly, the structure of fish skin varies enormously among species. In nearly all cases, however, it is possible to identify two major layers: an outer layer (epidermis) and an inner layer (dermis). These two layers are separated from the underlying muscle by the hypodermis (also called *subcutis*). Accessory structures associated with the skin include sensory receptors, scales, mucous glands, poison glands, luminous organs, and electric organs.

The epidermis can be divided further into two layers. An outer layer made up of interlocking stratified epithelial cells forms a tough barrier at the skin surface. Below this is the basal lamina layer, or stratum germinativum, which is composed of highly metabolic cells that differentiate into the various cell types found in the epidermis. Goblet cells produce mucus that is continually secreted at

the skin surface. To support this function there is a continual upward movement of differentiating goblet cells that begins at the basil lamina. The mucus produced by goblet cells serves a protective role and reduces the hydrodynamic resistance to swimming. Chemical irritation or physical stress will stimulate an increase in mucus secretion and bring about an increase in the number of differentiating goblet cells. Leydig cells also differentiate from the basal lamina but do not release their contents unless the epidermis is damaged. An alarm substance pheromone produced by Leydig cells acts to warn other fish of possible danger.

The dermis is also divided into two distinct zones, the upper stratum spongiosum and the lower stratum compactum. The stratum spongiosum contains fibroblasts, collagen, scale pockets, and pigment cells. The stratum compactum is made up of large collagen bundles attached at right angles to the skin surface. This architecture provides rigidity and allows the fish to swim without wrinkles forming at the skin surface. The stratum compactum also contains sense organs (touch and taste), nerves, and pigment cells. The dermal vascular system supplies the two dermal layers and the basal lamina of the epidermis. The dermis is connected to the underlying muscle by the hypodermis (subcutis), which is largely composed of connective tissue and fat cells.

Physiological functions of fish skin include respiration, ion exchange, and acid–base regulation. The skin of rainbow trout contributes to Ca^{+2} balance through active transport within the epithelial cells (Marshall et al., 1992) and to acid–base balance through Cl^{-}/HCO_3^{-} exchange across the extrabranchial epithelium (Ishimatsu et al., 1992). The skin of large fish (>100 g) consumes oxygen but in most species takes up only enough to satisfy the skin oxygen demand (Kirsch and Nonnotte, 1977; Nonnotte, 1981, 1984; Nonnotte and Kirsch, 1978). Based on morphometric and anatomical information, researchers have long suggested that cutaneous oxygen flux contributes significantly to total respiration in small larval fishes (McDonald and McMahon, 1977; McElman and Balon, 1980; Oikawa and Itazawa, 1985). Because the gill epithelium of both small and large fish consists of one or a few cell layers, its thickness does not change much with fish size. In contrast, skin thickness tends to decrease with decreasing fish size and in small fish may approach the thickness of the gill epithelium. In larval Chinook salmon (*Oncorhynchus kisutch*), as much as 80% of oxygen uptake takes place across the skin (Rombough and Moroz, 1990).

Dermal Absorption of Xenobiotics

Direct measurements of dermal uptake in rainbow trout and channel catfish were obtained by exposing fish confined in Plexiglas® chambers to a mixture of three chloroethanes (McKim et al., 1996). Although the rates at which both species approached steady state were similar to those observed in inhalation exposures to the same compounds, steady-state blood concentrations were much lower. A kinetic analysis of these data suggested that dermal uptake flux was about three to five times greater in catfish than in trout (Nichols et al., 1996). Some of this difference may have been due to differences in chemical diffusion rates at the experimental temperatures used for catfish (21°C) and trout (12°C); however, differences in skin anatomy and physiology, including the presence (trout) or absence (catfish) of scales were also likely factors. In addition, the analysis suggested that for adult trout dermal absorption would contribute 2 to 4% of initial uptake (dermal plus branchial) in a hypothetical waterborne exposure, but for catfish it would contribute 7 to 9%. Based on these findings, it appears that dermal uptake is a minor route of exposure in large fish, except perhaps for species that live in intimate contact with contaminated sediments.

In contrast, several studies have suggested that dermal uptake contributes substantially to total uptake of waterborne chemicals by small fish and juveniles of larger species. Tovell et al. (1975) measured anionic detergent uptake across the skin of small goldfish and found that 20% of total uptake occurred by this route. Saarikoski et al. (1986) obtained dermal uptake values for guppies exposed to a series of phenols, anisoles, and carboxylic acids. To distinguish between gill and skin absorption, fish were positioned into a hole cut in a rubber membrane separating two exposure chambers. Estimates of dermal absorption ranged from 25 to 40% of total absorption. Japanese medaka exposed to 2,2′,5,5′-tetrachlorobiphenyl in water accumulated considerably more chemical than could be explained by inhalation uptake (Lien and McKim, 1993). Similar results were reported for fathead minnows exposed to three chloroethanes (Lien et al., 1994). Lien and McKim (1993) suggested that dermal uptake should increase

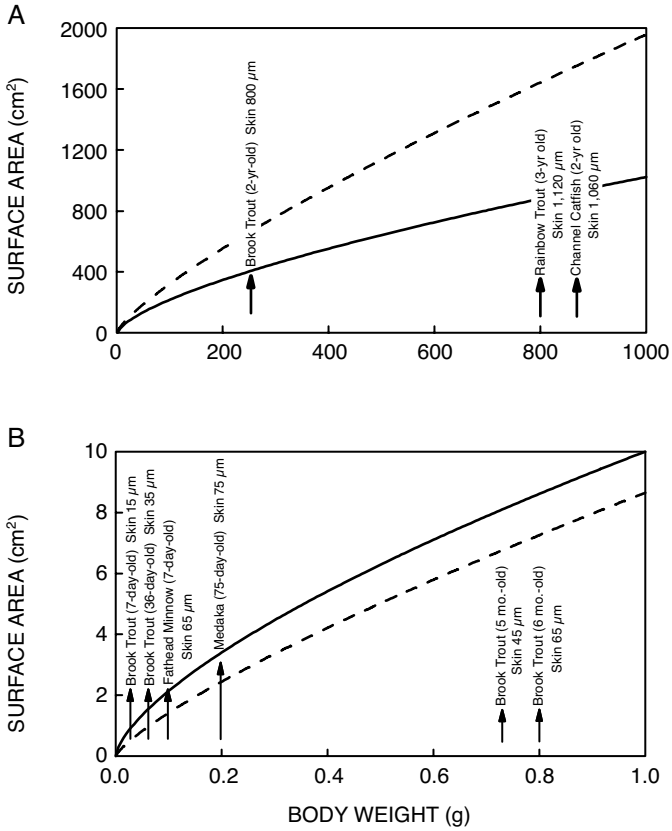

FIGURE 3.10 Skin (solid line) and gill (dashed lines) surface areas as a function of body weight. Arrows represent body weights of different species and life stages and their measured skin thickness (Rodney Johnson, personal communication). (A) Fish weighing up to 1000 g; (B) fish weighing less than 1 g.

in relative importance in small fish because of the way in which gill and skin surface areas scale to fish body weight. In smallmouth bass, gill surface area scales to body weight by a fractional exponent of about 0.78, consistent with the role of the gill in supporting metabolic activity (Price, 1931). Skin surface area scales instead to a body weight exponent of about 0.67 (Schmidt-Nielson, 1984). Using these values, Lien and McKim (1993) predicted that skin surface area approaches and may even exceed that of the gills in fish weighing less than 5 g (Figure 3.10). Reduced skin thickness and increased skin vascularization (Rombough and Moroz, 1990) also contribute to relatively greater dermal uptake in small fish.

Xenobiotic Distribution

Primary Determinants

A variety of processes act to distribute absorbed toxicants within fish. A portion of the absorbed compound may distribute to a site of action (organ, tissue, fluid) where toxic effects are expressed. Other sites serve as repositories for the chemical over short or long periods of time, while others are mobile and provide a means of transit within and out of the animal. During its residence in the body, a toxicant will redistribute continuously due to changes in chemical concentration gradients that drive xenobiotic movement.

Five primary factors control the distribution of xenobiotics from blood to peripheral tissues: (1) the physicochemical characteristics of the compound (e.g., pK_a, lipid solubility, molecular volume), (2) the concentration gradient between blood and tissues, (3) the ratio of blood flow to tissue mass, (4) the relative affinity of the chemical for blood and tissue constituents, and (5) the activity of specific membrane

transport proteins. For many compounds, movement out of blood and into tissues occurs by simple diffusion following a concentration gradient. When a compound diffuses rapidly across biological membranes, organ blood flow and the maintenance of a concentration gradient are primary determinants of distribution. In other cases, the rate of membrane diffusion may control chemical flux between blood and tissues. Chemical affinities for blood and tissue constituents can further influence the distribution process, both as an impediment to distribution (e.g., plasma protein binding) and as a means of facilitating uptake by creating a favorable tissue-to-blood concentration gradient. As discussed below, distribution processes controlled by simple diffusion or blood-flow rate generally exhibit first-order kinetics. Under these circumstances, the rate of chemical flux between blood and tissue is proportional to the magnitude of the concentration gradient. In contrast, membrane transport proteins often exhibit nonlinear (saturable) kinetics.

The distribution of a xenobiotic can also be viewed in terms of the fluid spaces that it occupies. Blood and extracellular and intracellular fluid spaces may individually or collectively define the distribution volume of a compound. This distribution is determined by: (1) binding to nondiffusing molecular species, and (2) the ability of the unbound compound to diffuse across biological membranes. If a xenobiotic is confined to plasma, low (perhaps unmeasurable) concentrations will be found elsewhere. If the same quantity of toxicant were distributed to interstitial fluid or total body water, the plasma concentration would be markedly lower.

Local Distribution

At early time points in an exposure, chemical distribution to tissues may be highly influenced by the route of administration. High concentrations in the skin following exposure to contaminated sediments, the gastrointestinal mucosa following dietary exposure, or muscle following an intramuscular injection are obvious examples. These distribution patterns are generally restricted to the absorption phase of the exposure.

Anatomical and physiological peculiarities of fish may also result in characteristic distribution patterns; for example, xenobiotics administered by intramuscular injection in the trunk muscle may be initially transported to the kidney. Venous blood from the trunk muscle collects in the caudal vein, which is part of the renal portal circulation in fish (Figure 3.1). Similarly, compounds taken up from the GIT or intraperitoneal cavity are transported first to the liver by the hepatic portal vein and then to the gills via the ventral aorta before distributing into the general circulation. The systemic availability of a compound taken up by this route may be influenced, therefore, by elimination pathways operating in the gut, liver, and gills. The fish anesthetic tricaine methane sulfonate (MS 222), for example, when given by intraperitoneal injection will not produce anesthesia because of its rapid metabolism in the liver and the ease with which both metabolites and parent compound diffuse out across the gills (Hunn and Allen, 1974). Anesthetic concentrations of MS 222 in arterial blood can only be achieved by maintaining high MS 222 concentrations in water.

The Circulation

Blood flow, expressed per unit of tissue mass, is a major determinant of the rate of chemical distribution to tissues. The tissues with the highest blood perfusion rates in fish are kidney, red muscle, pyloric ceca, intestine, spleen, and liver (Barron et al., 1987a). Tissues receiving an intermediate level of blood perfusion include the gonads, skin, and white muscle, while adipose tissue and bone are poorly perfused. The influence of blood flow on chemical distribution was demonstrated in rainbow trout exposed to linear alkylbenzene sulfonate (LAS) in water (Tolls et al., 2000). LAS concentrations in the internal organs (primarily kidney and GIT) and liver exceeded 80% of their steady-state values after 8 hours of exposure, while concentrations in the muscle and skin continued to increase until 78 hours.

Blood flow to some tissues may change substantially in response to physiological stimuli. An example of this phenomena is provided by the pre- and post-prandial intestine (Axelsson and Fritsche, 1991; Axelsson et al., 1989, 2000). Exercise (Neumann et al., 1983) and hypoxia (Cameron, 1975) altered blood-flow patterns in rainbow trout and arctic grayling (*Thymallus arcticus*), respectively. Stress,

exercise, and hypoxia related to netting and venipuncture were associated with changes in xenobiotic kinetics (Kleinow, 1991). It is likely that these changes were due in part to changes in tissue blood flow.

Ambient temperature affects both cardiac output and the pattern of blood flow to tissues. An increase in temperature was associated with an increase in cardiac output (Barron et al., 1987a). Blood flow as a percentage of cardiac output decreased in most organs, maintaining perfusion rates at a constant level. These changes were offset, however, by a redistribution of blood flow to white muscle, substantially increasing the perfusion rate of this tissue.

Another circulatory feature of fish with unknown but potential significance to xenobiotic distribution is the secondary circulation. This network of anastomosing vessels arises from the walls of the primary arteries and parallels the primary circulation of arteries, capillaries, and veins (Olson et al., 1986). The secondary circulation shares some characteristics with the mammalian lymphatic system, such as structural attributes of the vessels and restricted access for formed elements, but is more limited in its distribution. Tissues perfused by the secondary circulation include the gill filaments, skin, peritoneal lining, and oral mucosa. Limited studies suggest that the secondary circulation has a volume greater than that of the primary circulation and a turnover time of several hours (Steffensen and Lomholt, 1992). When the primary and secondary circulations are combined and expressed as a percentage of body weight, the total circulatory volume places teleost fishes well into the upper range for vertebrates.

Xenobiotic Transport and Binding in Plasma

Several transport modalities contribute to the circulatory distribution of xenobiotics. Although many compounds are transported in blood as freely dissolved forms, others may be transported in association with proteins, lipoproteins, or cellular components. Xenobiotics interact with these components by several mechanisms. Covalent binding usually restricts further distribution of the compound. In contrast, noncovalent ligand–protein interactions result in reversible binding that follows the law of mass action. When more than one binding interaction is possible, the distribution of a compound among binding proteins depends on both the binding affinity of each protein and their relative concentrations. Binding affinities may change with changes in ionic strength, pH, temperature, and protein conformation. As long as binding is reversible, however, an equilibrium will tend to be reestablished, providing an efficient means by which xenobiotics are transported and redistributed. Binding to plasma proteins can have a large effect on chemical distribution and clearance; for example, in trout, the plasma free fractions of parathion and paraoxon were determined to be 1.2 and 52.5%, respectively. Clearance rates determined for each compound were 21.4 and 3020 mL/hr/kg, respectively (Abbas et al., 1996).

Fish and mammals differ somewhat with regard to the total concentration of plasma proteins as well as qualitative properties of individual protein classes. Total protein concentrations in fish are generally much lower than those in mammals, possibly resulting in a reduced number of binding sites. In mammals, plasma albumin plays an important role in binding weak organic acids, and weak organic bases bind to plasma glycoproteins. It is clear, however, that some fish species either do not possess albumin at all, such as sharks and rays (Metcalf et al., 1999; Weisiger et al., 1984), or exhibit only very low concentrations (De Smet et al., 1998). The plasma albumin of rainbow trout has been described as "*para*-albumin" because of significant functional differences from the albumin of mammals (Perrier et al., 1977). Thus, the characteristic binding of xenobiotics to mammalian albumin and other plasma proteins may not directly extrapolate to fish; for example, low concentrations of the antibiotic sulfadimethoxine become highly (>90%) bound when added to rat plasma, but this binding saturates over a concentration range of 0.2 to 10 m*M* (Figure 3.11). In contrast, sulfadimethoxine binding in rainbow trout plasma ranges from 13 to 17% over the same concentration range, suggesting a nonsaturable, nonspecific interaction. This low degree of protein binding in trout facilitates the elimination of sulfadimethoxine and may result in a relatively larger apparent volume of distribution when compared with mammals (Kleinow and Lech, 1988). Similar differences between fish and mammals have been reported for the antimicrobial ormetroprim (Droy et al., 1990).

For other compounds, plasma binding in fish and mammals appears to be very similar; for example, the binding of 1-butanol, phenol, nitrobenzene, and pentachlorophenol was shown to correlate positively with chemical log K_{ow} in plasma obtained from both the rainbow trout and rat (Schmieder and Henry,

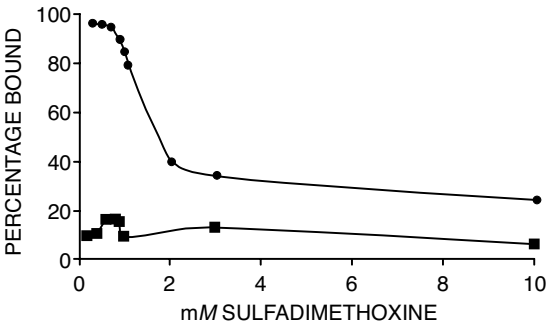

FIGURE 3.11 Plasma binding of sulfadimethoxine in rats (solid circles) and rainbow trout (solid squares) over a dosage range of 0.2 to 10 m*M*.

1988). This type of binding appears to be relatively nonspecific and nonsaturable and may be dominated by chemical interactions with lipid and lipoprotein. Similar relationships are obtained, however, when chemicals are added to solutions containing bovine serum albumin or human serum albumin (Figure 3.12). Using blood serum from mosquitofish (*Gambusia affinis*), Denison and Yarbrough (1985) found that the binding of lipophilic organochlorine insecticides increased with chemical polarity. Moreover, pretreatment of serum with endrin or aldrin substantially reduced the amount of subsequent DDT binding. Both of these observations are indicative of chemical binding to proteins.

Methylmercury in plasma binds reversibly to sulfur-containing molecules such as glutathione and the amino acid cysteine. The cysteine-bound form is of particular interest to toxicologists because it is transported by a neutral amino acid carrier system into sensitive tissues such as the brain (Kerper et al., 1992). In rainbow trout, however, 90% of whole-blood methylmercury is bound to hemoglobin in red blood cells (Giblin and Massaro, 1975). By limiting the amount of methylmercury available to interact with plasma molecules, this binding to hemoglobin becomes an important determinant of methylmercury kinetics and toxicity.

Heavy metals are transported in association with a variety of free proteins in plasma. Most of the cadmium in the plasma of brown trout and humans is bound to albumin or an "albumin-like" protein. Studies with carp, however, suggest that cadmium in plasma is primarily bound to a Mr 70,000 protein identified as transferrin (De Smet et al., 2001). The very low levels of "albumin" in carp plasma may account for this difference among species. Similarly, catfish serum proteins were shown to have much higher affinity and binding capacity for zinc than the primary transport protein (albumin) identified in other species (Bentley, 1991).

Tissue Affinity

Although chemical distribution at the beginning of an exposure is often determined by relative tissue blood flows, many chemicals redistribute over time in accordance with their relative affinity for tissue constituents. Lipophilic xenobiotics such as PCBs possess high affinity for tissues that have a high lipid content. Distributional differences among species may therefore occur due to different patterns of lipid deposition in organs and tissues; for example, Guiney and Peterson (1980) found that 60% of a dose of 2,2′,5,5′-tetrachlorobiphenyl in rainbow trout was contained in skeletal muscle and carcass, but in yellow perch 70% was contained in viscera and carcass (excluding skeletal muscle). These patterns were correlated with differences in the lipid content of each tissue, relative to that of other tissues. Similarly, Zitko et al. (1974) found that differences in muscle lipid content of Atlantic herring (*Clupea harengus*) and yellow perch correlated with differences in total PCB concentration.

Tissues that contain a large amount lipid may act as storage depots for lipophilic compounds. This storage may protect against adverse effects by isolating a chemical away from its sites of toxic action. Conversely, storage in these sites may prolong the overall residence time of a compound in the body and promote accumulation during chronic exposures. By reducing adipose lipid stores, starvation may result in a relatively rapid mobilization of accumulated chemical. When the rate of elimination from the

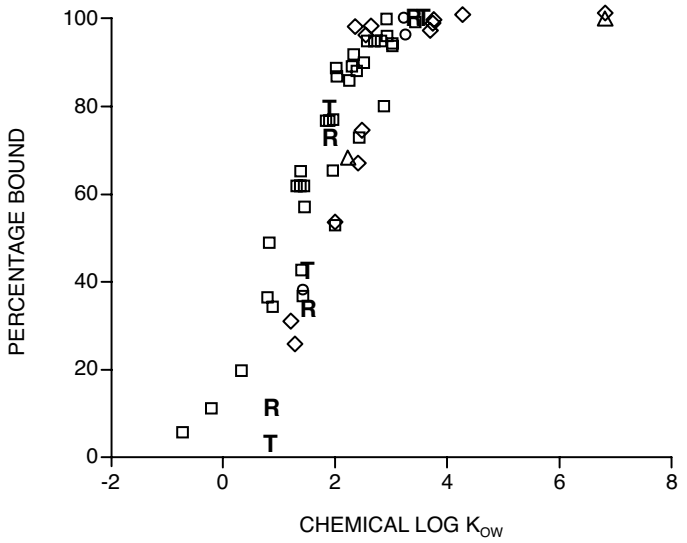

FIGURE 3.12 Chemical binding to plasma proteins as a function of chemical log K_{ow}. The symbols (T) and (R) represent values reported by Schmieder and Henry (1988) for trout and rat plasma proteins. Other symbols represent values summarized by the authors: open squares, bovine serum albumin; open circles, rat plasma; open triangles, shark plasma; open diamonds, human serum albumin. (Adapted from Schmieder, P.K. and Henry, T.R., *Comp. Biochem. Physiol.*, 91C, 413–418, 1988.)

whole animal is low, this starvation-induced decrease in whole-body lipid content may cause chemical concentrations in relatively lean tissues to increase substantially, even as whole-body chemical concentrations remain relatively constant (Gruger et al., 1975; Lieb et al., 1974).

Affinity considerations also determine the tissue distribution of lead during chronic exposures. In this instance, the accumulation of lead occurs because of its structural similarity to calcium. In fish, as in mammals, most of the accumulated lead is contained in bone (Camusso et al., 1995).

Lipid Mobilization and Xenobiotic Redistribution in Reproducing Fish

Female fish support egg development by mobilizing lipids from body stores. This lipid may be transferred to the growing egg mass or incorporated into larger energy storage molecules such as vitellogenin. Additional lipid may be mobilized in males and females to provide energy for spawning behaviors such as migration and nest defense (Jobling et al., 1998). In either case, xenobiotics may redistribute from fat storage depots to the developing gonads. To investigate this phenomena, Vodicnik and Peterson (1985) exposed female yellow perch to [^{14}C]-2,2′,5,5′-tetrachlorobiphenyl in water for 24 hours and then monitored tissue distribution and elimination for 5 months. Two weeks after exposure, 30% of chemical retained by fish was present in the developing ovaries. This value increased to 50% just prior to spawning, which occurred 4 months into the study. Similar studies with rainbow trout demonstrated the redistribution of [^{14}C]-2,2′,5,5′-tetrachlorobiphenyl to eggs and sperm (Guiney et al., 1979). Developmental stage may be an important determinant of xenobiotic transfer to eggs. When steelhead trout were given an intravascular dose of trifluralin (log K_{ow} 5), little or no chemical was transferred to mature eggs (Schultz and Hayton, 1997).

The percentage of an accumulated lipophilic contaminant that is redistributed to the developing gonads depends on several factors including: (1) the lipid content of the fish prior to gonad development, (2) the fraction of whole-body lipid content that is mobilized and incorporated into the gonads, and (3) the size of the gonads relative to total body weight. Niimi (1983) examined these features in five fish species: rainbow trout, yellow perch, smallmouth bass, white bass, and white sucker. Yellow perch were the leanest of these five species (5.1%) and transferred the greatest percentage of accumulated contaminants (25.5%) to their eggs. In contrast, rainbow trout had the highest starting lipid content (11.4%) and transferred the lowest percentage of contaminants to their eggs (5.5%). The high percentage of

contaminant transfer in spawning yellow perch was due to the large size of its egg mass (22.3% of body weight) and the fact that it transfers a high percentage of its limited whole-body lipid stores to the developing ovaries (27.1%).

Reproductive life history may also influence the redistribution of lipophilic contaminants in fish. The Chinook salmon, a semelparous (once-bearing) species, transfers most of its stored lipid into a single spawn of eggs. By comparison, iteroparous (multiple-bearing) lake trout invest a lower proportion of stored lipid into each reproductive effort. In a study of these two species in Lake Michigan, Miller (1993) found that female salmon transferred 28 to 39% of their accumulated PCB body burden to the eggs during a single spawn, but female lake trout eliminated 3 to 5% of whole-body PCBs during each of what may be several spawns.

Other Features of Distribution

In mammals, modifications to membranes within the eye and central nervous system limit the movement of many xenobiotics. Similarly, chemical uptake by the mammalian testis may be limited by the presence of active transport systems and high levels of biotransformation enzymes. Membrane structure and function within these tissues in fish are essentially unknown. Xenobiotic movements are also limited within the placenta and mammary gland of mammals, but these tissues have no counterparts in fish. An important consideration for fish and other poikilotherms is the impact of temperature on chemical distribution. Reductions in temperature lower cardiac output (Barron et al., 1987a); alter membrane composition (Crockett and Hazel, 1995); change tissue perfusion patterns (Barron et al., 1987a), xenobiotic fluid space distribution (Van Ginneken et al., 1991), and partitioning to storage tissues (Barron et al., 1987b; Karara and Hayton, 1989); and elicit a hypertrophic response in the intestine and liver (Das, 1967; Lee and Cossins, 1988). For many xenobiotics, an inverse relationship exists between environmental temperature and chemical retention (generally characterized using the terminal elimination half-life) (Bjorklund et al., 1992; Collier et al., 1978; Jacobsen, 1989; Kasuga et al., 1984; Kleinow et al., 1994; Salte and Liestol, 1983; Van Ginneken et al., 1991; Varanasi et al., 1981). These observations may be due in part to changes in distribution, although changes in the activities of chemical elimination pathways are likely to contribute.

Xenobiotic Elimination

Branchial Excretion

In teleosts, the most important route of elimination for neutral, water-soluble, low-molecular-weight chemicals is across the gills. Working with the Dolly Varden char (*Salvelinus malma*) in a split chamber system, Thomas and Rice (1981) showed that aromatic hydrocarbons with low to moderate lipid solubility (log K_{ow} 1 to 4) are eliminated across the gills at a greater rate than those with high lipid solubility (log K_{ow} 4 to 7). A similar finding was reported for goldfish exposed to a series of substituted phenols with log K_{ow} values ranging from 1 to 5 (Nagel and Urich, 1980). Erickson and McKim (1990b) compiled data from several studies involving guppies (Bruggeman et al., 1984; Gobas et al., 1989; Konemann and van Leeuwen, 1980; Opperhuizen et al., 1985) and found that elimination rates declined linearly with chemical log K_{ow} across a wide range (3 to 8) of values (Figure 3.13). These rate calculations were based, however, on retained chemical residues and may have reflected elimination by branchial and non-branchial routes.

Current models of chemical flux at fish gills (Erickson and McKim, 1990b) suggest that this dependence of branchial elimination on chemical lipophilicity (or hydrophobicity) is due largely to chemical binding in blood. The effect of this binding is to lower the diffusion gradient across the gill epithelium by reducing the concentration of chemical in blood that is in a "free" (diffusing) form. Direct tests of this hypothesis for high log K_{ow} compounds are difficult to perform due to very low chemical concentrations in expired water and the tendency of these compounds to adsorb to tubings, glassware, and other experimental apparatus. Dvorchik and Maren (1972) injected dogfish sharks (*Squalus acanthias*) with [^{14}C]-DDT and collected samples of expired water but were unable to detect any radioactivity eliminated by this route. More recently, Fitzsimmons et al. (2001) developed a method using continuous column extraction of expired water, coupled with high-resolution mass spectrometry, to measure

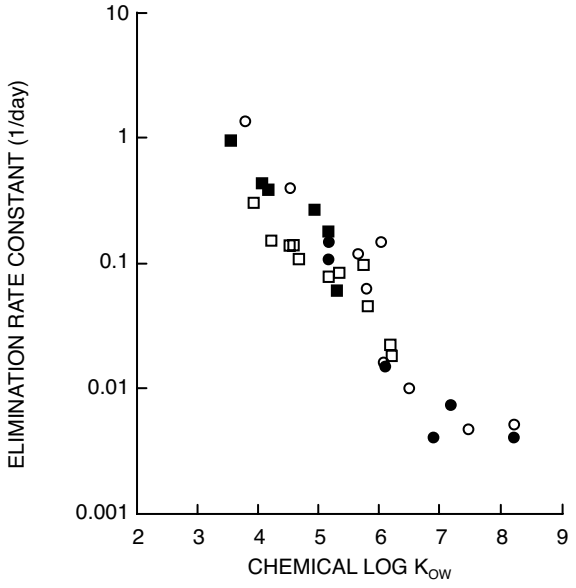

FIGURE 3.13 Dependence of elimination rate in guppies on chemical log K_{ow}: solid squares, chlorinated benzenes; solid circles, chlorinated benzenes/biphenyls; open squares, chlorinated benzenes/naphthalenes; open circles, brominated benzenes/biphenyls and chlorinated biphenyls. (Adapted from Erickson, R.J. and McKim, J.M., *Aquat. Toxicol.*, 18, 175–198, 1990.)

branchial elimination of four PCBs with log K_{ow} values ranging from 5.8 to 8.2. All four compounds were eliminated at low, but measurable rates. An analysis of these data suggested that a near-equilibrium condition was established between chemical in venous blood entering the gills, including dissolved and bound forms, and dissolved chemical in expired branchial water. At the other end of the spectrum, organic chemicals such as detergents (Schmidt and Kimerle, 1981) and drugs with low (<1) log K_{ow} values, or those that are ionized at the pH of fish blood (Maren et al., 1968), tend not to be eliminated across the gills but are eliminated in urine or bile.

Biliary and Fecal Elimination

Several hepatic and intestinal processes in fish may contribute to the elimination of xenobiotic chemicals. Compounds incompletely absorbed from the diet will be eliminated in feces. The absorbed fraction will be transferred to the liver and subjected to a variety of elimination pathways, including biotransformation and biliary secretion. Parent compounds and their metabolites in bile may be reabsorbed or expelled with the feces (Statham et al., 1976). Lipophilic xenobiotics that are poorly metabolized or cleared by renal and biliary routes can be transferred from blood to the intestinal lumen (Ingebrigtsen and Solbakken, 1985; Ingebrigtsen et al., 1988; Kleinow et al., 1996). Passive diffusion is an important mechanism for this process, although active transport (Leu and Huang, 1995) and exfoliation of intestinal cells may contribute.

Contribution of the Liver

The liver in fishes is a major site for amino acid homeostasis, glucose regulation, lipid processing, hormone metabolism, and the production of numerous plasma constituents, including albumin-like proteins and fibrinogen (Hinton et al., 2001). The liver also plays an important role in the excretion of xenobiotic compounds. The excretion of xenobiotics by the hepatic parenchyma depends on chemical uptake by hepatocytes, the formation and secretion of bile, and the transport of these compounds into bile. In this excretory process, hepatic and intestinal biotransformation often plays a pivotal role by producing polar anionic metabolites that are readily acted upon by the transport mechanisms of the liver.

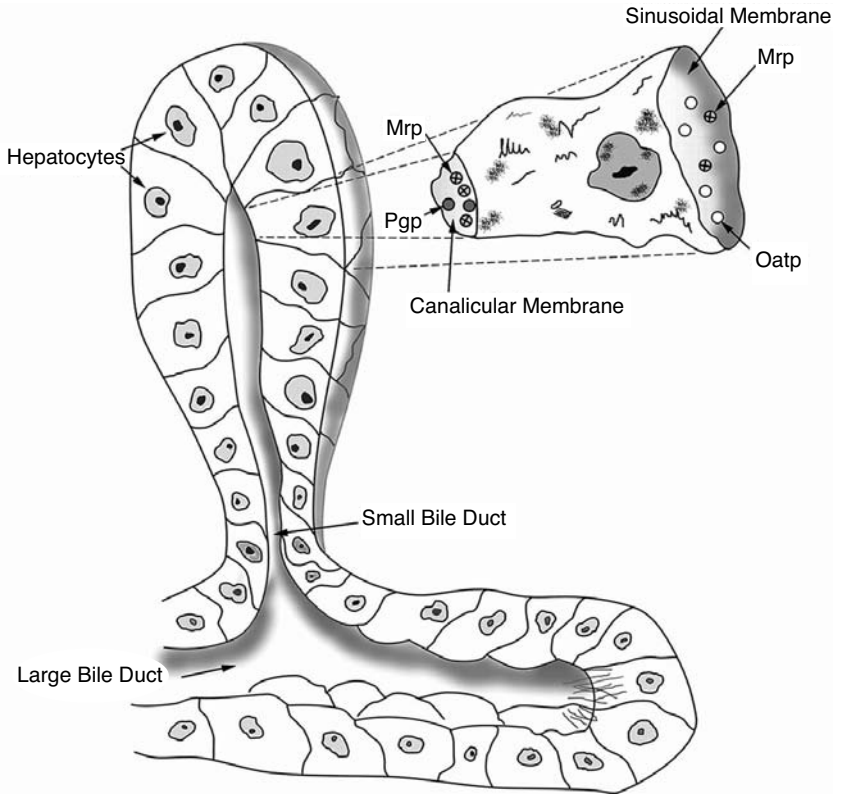

FIGURE 3.14 Hepatic tubule of the fish liver showing the origins of the biliary system and locations of xenobiotic transporters. Efflux transporter isoforms of the multidrug resistance-associated protein family (Mrp) are located on both the sinusoidal and canalicular membranes. P-glycoprotein (Pgp) is localized to the canalicular membrane, and isoforms of the organic anion transporter polypeptide family (Oatp) are located on the sinusoidal membrane.

A variety of structural arrangements have been described for the liver and biliary tree of fish. In general, fish do not possess the lobular structure and zonally oriented portal tracts observed in mammalian livers (Eurell and Haensly, 1982; Hampton et al., 1989; Hinton and Couch, 1998; Robertson and Bradley, 1992; Schar et al., 1985). Instead, the hepatocytes are commonly arranged into tubular elements. In rainbow trout, hepatocyte apices are directed toward the center of the tubule (Figure 3.14). At the center of the tubule, plasma membranes of adjacent cells form bile canaliculi. Where a biliary space exists, the biliary epithelial cells form junctional complexes with each other, and the hepatocytes on either side form a wall of the biliary passageway. These biliary passageways lead in turn to the cholangioles, small bile ducts, and large bile ducts. The contralateral or basal side of each hepatocyte faces a blood-filled sinusoid surrounding the tubule. Individual tubules curve, branch, and anastomose to form a complex network of parenchyma.

In salmon, multiple hepatic bile ducts empty into an elongated major duct that runs along the posterior ventral margin of the liver. The cystic duct branches from the major bile duct and serves to divert bile to the gallbladder. Bile originating from the major bile duct and gallbladder is collected in the common bile duct and empties into the proximal intestine just posterior to the pyloric curvature of the stomach. Some fish species, including members of the cod family, do not possess a gallbladder. It is thought that for these species bile is secreted continuously into the intestine.

Studies with mammals have shown that the excretory function of the liver and bile is greatly facilitated by the activity of specialized efflux transport proteins, including multidrug resistance-associated proteins (Mrps) and P-glycoprotein (Pgp) (Ayrton and Morgan, 2001; Keppler and Konig, 2000; Yamazaki et al., 1996). Mrps and Pgp belong to the highly conserved superfamily of ATP-binding cassette (ABC) transporters, which have been identified in organisms ranging from yeast to humans. Both groups of

transporters are plasma-membrane-bound export pumps; however, their amino acid sequence identity and substrate specificity differ.

At least nine forms of the Mrp subfamily have been identified in humans. Some, such as MRP2, MRP3, and MRP6 (capital letters indicate that these are human gene products), are primarily expressed in the liver, kidney, and GIT. Others, such as MRP1, MRP4, MRP5, MRP7, and MRP8, occur in a variety of tissues, including the prostate, testis, ovaries, pancreas, and lung, as well as the blood–brain and blood–testis barriers. Most Mrps reside on the basolateral side of the plasma membrane, although MRP2 has been localized to the apical side in epithelial tissues. Mrps actively transport organic anions into bile and urine. Substrates for these transporters include xenobiotics conjugated with sulfate, glutathione (GSH), and glucuronic acid. Several Mrps have also been shown to transport endogenous molecules such as cyclic nucleotides, nucleoside analogs, and free GSH.

P-glycoprotein is an apical transporter of weakly cationic compounds and is expressed in a variety of secretory cell types. Substrates for Pgp (also referred to as Mdr1) generally possess a molecular weight between 350 and 1000, contain two planar rings, and exhibit a moderate degree of hydrophobicity. Known substrates for Pgp include steroids, lipids, and peptides, as well as a variety of xenobiotics and drugs such as adriamycin and vinblastin. Pgp appears to contribute to the regulation of some steroid hormones. This regulation is achieved by the secretion of hormones from sites of synthesis, such as the adrenal gland, and the modulation of hormone concentrations in target tissues such as the brain and testis.

A third class of transporters, organic anion transporter polypeptides (Oatps), are found in the liver, as well as in other tissues. Members of the Oatp family reside on the sinusoidal (basolateral) membrane of hepatocytes and transport negatively charged compounds. Unlike the Mrps and Pgp, Oatps function as influx transporters.

Localization studies and functional experiments suggest that homologs to these transport proteins operate in fish liver as well. Using immunohistochemical methods, homologs of mammalian Pgp have been shown to exist in the bile canaliculi of several species (Curtis et al., 2000; Hemmer et al., 1995; Kleinow et al., 2000). Studies with prototypical Pgp substrates have demonstrated the existence of an ATP-dependent transporter function in both trout and channel catfish (Doi et al., 2001; Sturm et al., 2001). Ballatori et al. (1999) used competitive inhibition methods as well as transport inhibitors to demonstrate that uptake of lucifer yellow (a model organic anion) by skate hepatocytes is carrier mediated. Additional studies with the skate suggest that glutathione conjugates are secreted into bile by an efflux transporter with Mrp-like activity, located on the canalicular membrane (Rebbeor et al., 2000).

Fish excrete a wide variety of xenobiotics in the bile. A partial listing of these compounds includes antibiotics (Samuelsen et al., 1995), insecticides (Lech et al., 1973), dyes (Plakas et al., 1992a), herbicides (Schlenk and Moore, 1993), polyaromatic hydrocarbons (Collier and Varanasi, 1991), metals (Grosell et al., 2001), and natural products (Sahin et al., 1996). Early studies with rainbow trout showed that chemical concentrations in bile may be much higher than those in the surrounding water (Lech et al., 1973; Statham et al., 1976). Guarino and Lech (1986) summarized the results of studies with fish exposed to more than 40 drugs and chemicals of varying molecular weight. The data were examined to determine bile/plasma concentration ratios and the percentage of compound eliminated in bile or urine. Bile/plasma concentration ratios were greater than 1 for over 85% of compounds examined, suggesting that fish were capable of concentrating xenobiotics in bile relative to plasma. The relative importance of biliary excretion as a route of elimination increased with molecular weight, although other factors such as the charge on the molecule were important. Generally, chemicals that are highly polar and have molecular weights greater than 600 are excreted largely in bile (Gingerich et al., 1977; Schmidt and Weber, 1973). Compounds of intermediate molecular weight (300 to 600) and polarity may be eliminated in bile as well as by other routes (Allen et al., 1979; Hunn and Allen, 1975; Kobayashi et al., 1977). From these limited datasets we may conclude that the same criteria (molecular weight and polarity) that apply to biliary excretion in mammals are operative in fish.

Metabolic and Physiological Impacts

Biotransformation may substantially increase biliary elimination of xenobiotics by increasing their solubility and creating conjugated compounds that are substrates for active transport systems. Stehly and Hayton (1989c) examined the metabolism and biliary excretion of pentachlorophenol in several fish

species and found that the amount of metabolites in bile was species dependent. In each case, however, the bile was selectively enriched in glucuronide conjugates, and all of the metabolites in water were sulfate conjugates. These findings illustrate the potential selectivity of elimination pathways for metabolites as well as the inadvisability of defining metabolite profiles based solely on either bile or urine samples. Similar considerations apply to the elimination of some metal–ligand complexes. In the little skate (*Raja erinacea*) and dogfish shark, biliary elimination of methylmercury, which binds to glutathione and other sulfur-containing biomolecules, was reduced by inhibiting the biosynthesis of glutathione or its excretion in bile (Ballatori and Boyer, 1986).

Once secreted into the intestine, biliary conjugates may undergo enzyme-mediated hydrolysis, liberating the parent compound for reabsorption and enterohepatic recirculation (Collicutt and Eales, 1974; James, 1987; Fricker et al., 1997; Layiwola et al., 1983; Schultz et al., 2001). These processes modulate the amount of metabolites eliminated via the feces. In addition, *in situ* intestinal studies in fish suggest that conjugated metabolites of highly lipophilic compounds such as benzo(*a*)pyrene (benzo(*a*)pyrene 9-sulfate and benzo(*a*)pyrene 9-glucuronide) may be absorbed intact from the GIT (James et al., 1996). The uptake these conjugates appears to be due to their relatively low molecular weight and appreciable lipophilicity.

Secretion of bile into the intestinal tract is stimulated by nutritional and digestive signals. Due to the episodic nature of bile secretion and potential for xenobiotic reabsorption within the intestine, it is difficult to assess the contribution of biliary excretion to the overall process of elimination. The gallbladder of a recently fed fish is generally void of bile. By necessity, therefore, chemical concentrations in bile are generally determined using fasted animals.

Techniques for continuous collection of bile in unanaesthetized rainbow trout were described by Schmidt and Weber (1973), Gingerich et al. (1977), and Sanz et al. (1993). Similar methods have been developed for the skate (*Raja erinacea*) and dogfish shark (*Squalus acanthias*) (Boyer et al., 1976a,b,c). Basal bile flows measured using these techniques range from 50 to 200 μL hr^{-1} kg^{-1} in rainbow trout, 30 to 70 μL hr^{-1} kg^{-1} in the skate, and 80 to 110 μL hr^{-1} kg^{-1} in the dogfish. Bile flow rates in mammals may be 50 times greater than these values (Klaassen and Plaa, 1967). Differences in the rates of biliary elimination of bromosulfophthalein between rainbow trout and rats were found to be well correlated with differences in bile flow rate (Gingerich et al., 1977). This finding suggests that bile formation rate limits the rate of bromosulfophthalein excretion in bile. Fish have been shown to efficiently concentrate a large number of xenobiotics in bile, leading to the suggestion that bile can be used as a tool for monitoring environmental chemical exposures (Statham et al., 1976). This concentrating effect may be a result of slow bile flow relative to the secretion of xenobiotics into bile.

In rainbow trout, the biliary excretion of two organic anions, phenolphthalein (Curtis, 1983) and taurocholate (Curtis et al., 1986; Kemp and Curtis, 1987) increased with an increase in acclimation temperature. Similar results were obtained for a polyaromatic hydrocarbon, benzo(*a*)pyrene (Curtis et al., 1990). Hepatic blood flow has the potential to limit biliary clearance and is likely to increase with increased temperature (assuming that hepatic blood flow as a proportion of cardiac output remains constant). In the case of phenolphthalein, however, biliary elimination appeared to saturate at the two lower temperatures tested (Curtis, 1983). The mechanistic basis for these observations may be related, therefore, to changes in saturable processes responsible for chemical transport from blood to bile as well as the rate of chemical delivery to liver.

Urinary Excretion

Fish kidneys perform two major functions: (1) ion and water balance, and (2) excretion of endogenous and exogenous solutes. The diversity of kidney structure–function across species is enormous, but basic features are shared by both freshwater and saltwater teleosts. This section provides an overview of fish kidney function with an emphasis on mechanisms responsible for renal excretion of xenobiotic chemicals. Reviews of kidney structure–function in fish are provided elsewhere (Hickman and Trump, 1969; Pritchard, 1981; Nishimura and Imai, 1982; Pritchard and Miller, 1993; Pritchard and Renfro, 1984).

The functional unit involved with urine formation in the freshwater fish is the kidney (Figure 3.15), consisting of an encapsulated capillary network called the *glomerulus* and a tubule lined with epithelial

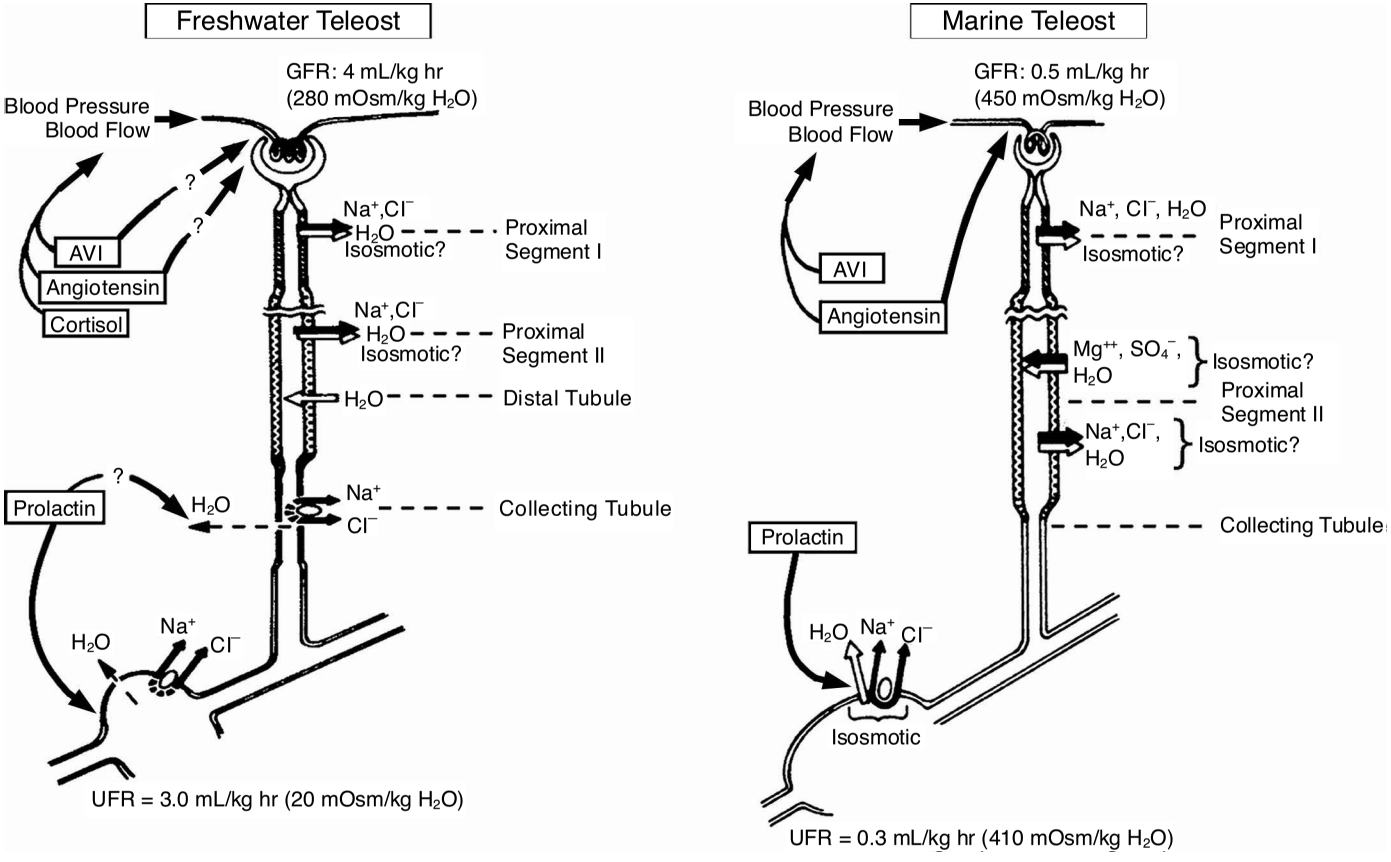

FIGURE 3.15 Renal structure, proposed handling of water and NaCl, and effects of hormones in freshwater and marine teleosts. Solid and open arrows indicate active and passive movements, respectively. GFR, glomerular filtration rate; UFR, urine flow rate. Broken straight and U-shaped arrows indicate low permeability to water and possible Na and Cl cotransport, respectively. (Adapted from Nishimura, H. and Imai, M., *Fed. Proc.*, 41, 2355–2360, 1982.)

cells, as well as the urinary bladder. The kidney tubule can be subdivided further into two proximal segments (I and II): a distal tubule and a collecting tubule. The functional kidney unit of saltwater fish is similar to that of freshwater fish but lacks the distal tubule. In some saltwater species, the glomerulus may also be absent.

Because they are hyperosmotic with respect to their environment, freshwater fish must eliminate large volumes of dilute urine and retain salt (principally NaCl). High urine flow rates are supported by a high glomerular filtration rate (GFR), which is controlled by hormones (arginine vasotocin, angiotensin, and cortisol) that act primarily on blood pressure. Additional control of GFR may exist at the local level via the recruitment of glomeruli. Epithelial permeabilities to water within the collecting tubule and bladder are controlled in part by the hormone prolactin and tend to be very low. In contrast, saltwater teleosts are hypoosmotic with respect to the environment and must overcome dehydration by drinking seawater and absorbing water and minerals (primarily monovalent ions) through the gut. Excess salts are eliminated across the gills (both mono- and divalent ions) and kidney (primarily divalent ions), and water loss is minimized by producing small quantities of relatively concentrated urine. Divalent ions that are not absorbed within the gut (primarily Mg^{2+} and SO_4^{2+}) are eliminated in feces. The GFR in saltwater fish is much lower than that of freshwater fish. Urine osmolarity is maintained by an exchange of divalent and monovalent ions within the proximal tubule. Water is then reabsorbed with NaCl in the bladder. Because it comprises up to 40% of total tubular surface, the proximal tubule of saltwater fish has been used extensively for both *in vitro* and *in vivo* studies of kidney function (Kinter, 1966, 1975; Miller and Pritchard, 1997; Pritchard and Miller, 1993; Pritchard and Renfro, 1984).

The mechanisms that have evolved for handling ions and water in the fish kidney also contribute to the excretion of endogenous and exogenous organic solutes. The first step in urine formation is ultrafiltration of plasma through the capillary network of the glomerulus. In the lumen of the tubule, solutes may be reabsorbed back into plasma or added to the filtrate by secretion of free chemical from the plasma across the renal epithelium. The final composition of urine is determined by characteristics of the epithelium in both the tubule and bladder (Miller, 1987).

Two important attributes of organic solutes that determine the effectiveness of their renal excretion are (1) molecular size, and (2) plasma binding. Molecules with a radius of less than 20 Å are completely filtered at the glomerulus, those with a radius between 20 and 42 Å are partially filtered, and those with a radius greater than 42 Å are retained in plasma (Miller, 1987). Molecular size also limits renal tubular secretion. In fish as in mammals, chemicals with molecular weights greater than 500 tend to be secreted in the liver rather than the kidney (Pritchard and Renfro, 1984). Plasma binding is important because glomerular filtration and tubular secretion act primarily on free or unbound chemicals in plasma. Because they bind to plasma proteins, lipophilic compounds are generally retained by plasma as it flows though the glomerulus. Lipophilic chemicals contained within the glomerular filtrate may be reabsorbed from the tubule. This is particularly true of saltwater fish due to the concentrating effect of water reabsorption.

Some of the xenobiotics that fish are exposed to exist as organic anions or cations. Other compounds are initially taken up as neutral species and then converted to anions and cations by various biotransformation pathways. In all vertebrates studied to date, including fish, anionic and cationic secretory systems located in the proximal tubule transport charged compounds from plasma into the tubule urine (Pritchard and Miller, 1993). The caudal vein in fish drains into the kidney, providing a substantial supply of portal blood. Most of this portal blood bathes the kidney tubules and contributes to the renal secretory system (Pritchard and Renfro, 1984). Overall, up to 80% of the cardiac output flows through the fish kidney, as compared with about 20% in mammals.

Tubular Anion Secretion

The organic anion secretory system uses metabolic energy to move substrate molecules into the tubule epithelial cells against their electrochemical gradient (Figure 3.16). This mechanism is carrier mediated, saturable, and inhibited competitively by other substrates (Pritchard, 2001). Organic anion secretion is a three step process: (1) ATP is hydrolyzed to drive the Na^+ pump and create an inward Na^+ gradient; (2) the Na^+gradient into the cell drives the uptake of α-ketoglutarate (αKG^{-2}), thereby creating an outward

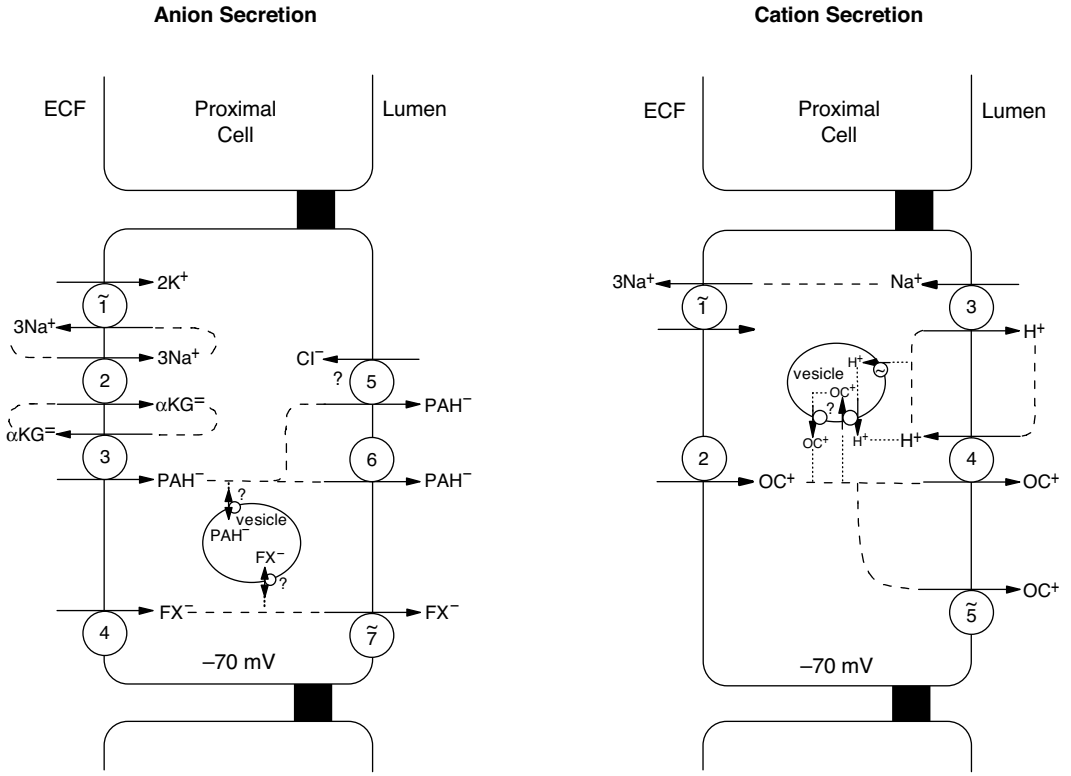

FIGURE 3.16 Mechanisms for renal secretion of organic anions and organic cations. Open circles represent carrier proteins, the symbol ~ within a circle indicates an ATPase, and arrows show the preferred direction of transport. (A) *Organic anion secretion:* The transport of *p*-aminohippuric acid (PAH^-), a prototypical organic acid, is coupled to ATP hydrolysis and the movement of α-ketoglutarate (αKG^{-2}) (components 1 and 3). Larger organic acids, such as fluoroscein-methotrexate (FX^-), may enter the proximal tubule cell by a Na-independent path (4) or simple diffusion. Intracellular organic anions may diffuse to the luminal membrane, bind to macromolecules, or be transported into vesicular structures. Uptake into vesicles is carrier mediated but the mechanisms are unknown. Efflux to the lumen may occur by facilitated diffusion (6) or through the activity of a drug-transporting ATPase such as Mrp2 (7). The luminal anion exchanger (5) is primarily responsible for the reabsorption of urate but may play a role in the secretion of other anions. (B) *Organic cation secretion:* Facilitated transport of an organic cation (OC^+) across the basolateral membrane is favored by a negative membrane potential (2). Some of the OC^+ may be sequestered in vesicles; the rest is transported to the lumen by two pathways. The first pathway involves H^+/OC^+ exchange (4), which is driven by a pH gradient (3) that is coupled to the Na^+ pump (1). The second pathway involves the multidrug transport ATPase MDR (5). (Adapted from Pritchard, J.B., in *The Textbook of Nephrology*, 4th ed., Massry, S.G. and Glassock, R.J., Eds., Lippincott Williams & Wilkins, Baltimore, MD, 2001, pp. 93–97.)

αKG^{-2} gradient; and (3) the organic anion moves into the cell in exchange for the outward movement of αKG^{-2}. Efflux from the epithelial cells into the tubule may occur by facilitated diffusion. Other compounds are transported across the luminal membrane of the cell by the drug-transporting ATPase Mrp2 (Miller and Pritchard, 1997).

Tubular Cation Secretion

Renal cation secretion is also a multistep process (Figure 3.16). In contrast to anion secretion, however, chemical movement into the epithelial cell (by facilitated diffusion) is energetically favored because of the strong negative charge on the cell interior. Movement into the lumen of the tubule is opposed by the membrane potential and must be tied to the hydrolysis of ATP (Pritchard, 2001). As with anion secretion, the cationic secretory system is highly susceptible to substrate competition.

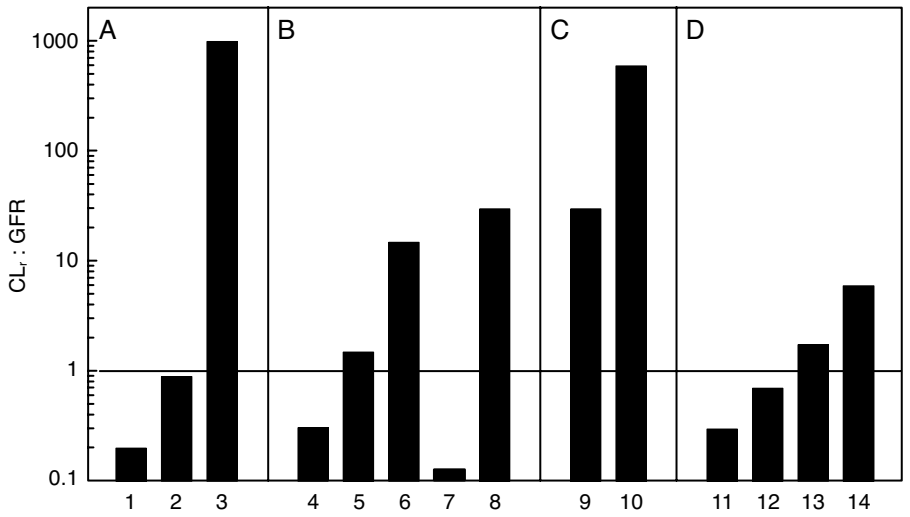

FIGURE 3.17 Influence of tubular transport, biotransformation, and plasma binding on renal excretion in marine teleosts (A–C) (Pritchard and Renfro, 1984) and freshwater teleosts (D) (McKim et al., 1999b). Data for each compound are expressed as the ratio of renal clearance (CL_r) to glomerular filtration rate (GFR). (A) *Reference compounds:* (1) glucose, reabsorbed by active transport; (2) 2-deoxyglucose, with no net tubular transport; (3) *p*-aminohippuric acid, a prototypical substrate for active anion secretion pathway. (B) *Effect of metabolism:* (4) benzo(*a*)pyrene (BaP), reabsorbed passively; (5) BaP-7-phenol conjugates (largely glucuronide), limited anion secretion; (6) BaP-7,8-dihydroxydiol conjugates (largely sulfate), a better substrate for anion secretion; (7) DDT, passive reabsorption; (8) DDA, effective tubular secretion. (C) *Effect of plasma protein binding:* (9) DDA, plasma binding of 97%; (10) 2,4-D, plasma binding of 70%. DDA and 2,4-D are transported similarly *in vitro* (without binding). (D) *Effects of reabsorption and metabolism:* (11) hydroquinone, strong passive reabsorption; (12) phenol, slight passive reabsorption; (13) phenyl glucuronide, limited anion secretion; (14) phenyl sulfate, better substrate for anion secretion.

Renal Clearance

The mechanisms responsible for renal excretion of chemicals can be distinguished by dividing measured renal clearance rates (CL_r) by the GFR (McKim et al., 1999b; Pritchard and Renfro, 1984). CL_r is the volume of plasma completely cleared of chemical by the kidney in a given period of time and is calculated by dividing the excretion rate of chemical in urine by the chemical concentration in plasma at the midpoint of a sampling period. The GFR is the total amount of plasma filtered by the kidney and may be estimated using an *in vivo* [^{14}C]-polyethylene glycol method (Beyenbach and Kirschner, 1976). Figure 3.17 shows CL_r-to-GFR ratios for several compounds in both freshwater and saltwater fish. A CL_r-to-GFR ratio less than 1 suggests that a chemical is reabsorbed within the kidney tubule, a ratio of 1 indicates no net tubular transport, and a ratio greater than 1 signifies active secretion into the tubular fluid. Chemical binding in plasma can reduce renal tubular secretion as well as chemical filtration at the glomerulus. Generally, however, the effect of plasma binding on CL_r is greater than that on GFR, decreasing the ratio of CL_r to GFR. For a given mechanism of renal excretion, CL_r-to-GFR ratios in saltwater fish tend to exceed those of freshwater fish. This pattern appears to be due primarily to higher GFR values in freshwater fish.

Dermal Excretion

The extent to which chemicals and their metabolites are eliminated across the skin is poorly known; however, anatomical and physiological factors that promote chemical uptake across the skin of some fish species (e.g., high degree of vascularization and large ratio of skin area to gill area) also might be expected to facilitate chemical elimination. Lead and cadmium diffuse across the skin of juvenile rainbow trout and into the mucous layer, which is then sloughed from the fish (Varanasi and Markey, 1978). Naphthalene was recovered from the mucus of rainbow trout soon after it was administered by

intraperitoneal injection or dietary exposure (Varanasi et al., 1978). At later time points, only naphthalene metabolites were found. Whether or not these metabolites were formed in skin prior to their diffusion into mucus was not investigated. Using an *in vitro* skin strip preparation, Ali et al. (1987) showed that the skin of the African catfish (*Clarias gariepinus*) exhibits both phase I and phase II metabolic activity toward some endogenous steroids.

Elimination Via the Gametes

The redistribution of lipophilic chemicals from somatic lipid stores to developing gametes was described earlier in this chapter. Compounds for which this phenomenon has been demonstrated include anthracene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), DDT and its metabolites, PCBs, dibenzofurans, mirex, heptachlor epoxide, toxaphene, chlordane, and dieldrin (Kleinow et al., 1999). Additional studies have demonstrated the accumulation of zinc, lead, mercury, and cadmium in gametes of several fish species (Morrison et al., 1985). Vitellogenin binds several micronutrient metals, including iron, copper, and zinc; however, little is known about its role in transporting pollutant metals to developing oocytes (Kleinow et al., 1999).

The triggers for gamete-based elimination are gonadal maturation and reproductive behavior. These events may occur only seasonally for some fish species and year-around for others. Spawning may consist of a single event or multiple events (fractional spawners) occurring over a short or extended time interval. Although spawning eliminates xenobiotics from the animal, reducing the whole-animal contaminant load, whole-body chemical concentrations may increase (Tietge et al., 1998), decrease (Guiney et al., 1979; Niimi, 1983; Vodicnik and Peterson, 1985), or stay the same (Niimi, 1983), depending on the chemical concentration in gametes relative to the rest of the body. If the contaminant concentration in the gametes is lower than that of the rest of the fish, gamete release actually increases the chemical concentration in the remaining tissues (expressed on a whole-body basis). The reverse is true if the contaminant becomes highly concentrated in the gametes, relative to the rest of the animal.

The importance of maternal transfer in viviparous species is currently unknown. Wourms et al. (1988) estimated that, among teleosts, live-bearing strategies have developed in only 2 to 3% of species; however, two of these species, the mosquitofish and guppy, have been used extensively in aquatic toxicology research. To the extent that an exchange of blood occurs between female fish and the developing young (as in Poeciliidae), it can be speculated that maternal transfer of chemicals to offspring occurs in some live-bearing species. In species for which most early development takes place after the egg has left the follicle (Embiotocidae and live-bearing sharks), the opportunity for maternal transfer may be reduced.

Toxicokinetic Modeling

The uptake and disposition of xenobiotic chemicals in fish have been described quantitatively using both compartmental and physiologically based modeling approaches. Compartmental models are comprised of one or more compartments, each of which represents tissues and organs with similar kinetic properties. These compartments do not generally correspond to any particular anatomical or physiological entity. Physiologically based models also represent the fish using a set of compartments. The structure of the model, however, is based on anatomical, physiological, and biochemical information for the species of interest. The compartments in a physiologically based model often correspond to specific tissues and organs, although kinetically similar tissues may be lumped to form a single compartment. In a compartmental model, parameter values and the number of compartments are determined by nonlinear regression analysis of measured kinetic data. Physiologically based models may, in theory, be developed in the absence of experimental data. In practice, however, measured values are often required to fit a small number of critical parameters.

Both modeling approaches may be used to project toxicokinetic behavior beyond the experimental dataset on which the model is based. Using compartmental models, this may be done by determination of model parameter values for fish of different sizes or for different values of an environmental variable such as temperature. Empirical relationships that relate model parameters to fish size or an environmental

variable are then developed and used to estimate model parameter values for other sizes of fish or other values of the environmental variable. For physiologically based models, prediction of toxicokinetic behavior is accomplished by appropriate adjustments to model parameters such as organ and tissue masses, blood flows to organs and tissues, and water flow across the gills.

The following text provides an overview of clearance concepts common to both compartmental and physiologically based toxicokinetic modeling approaches. These two modeling approaches are then described in detail. Both have advantages and disadvantages with respect to their utility and requirements for development. Guidance on the application of each approach is given in separate summaries. A section on noncompartmental modeling is also provided. Noncompartmental models are used extensively in mammalian pharmacology and toxicology and offer important advantages when using sparse datasets.

Clearance Concepts

Toxicants taken up by fish may be eliminated by several pathways, including metabolism and excretion into bile, urine, and respiratory water. In some cases, elimination rates are extremely slow and chemicals are retained for days, weeks, or even months after the fish are transferred to a clean environment (Niimi and Oliver, 1983). Alternatively, elimination pathways may clear >99% of an absorbed dose within just a few hours. In either case, elimination may be characterized by an *elimination clearance* (CL). The CL is defined as the rate of toxicant elimination (dX/dt) divided by the concentration in the reference region (C):

$$CL = (dX / dt)/C \tag{3.6}$$

and has units of flow (e.g., L/hr or mL/min). Normalization to body size (weight or surface area) may also be appropriate, particularly when averaging values from fish of different sizes. The CL is the sum of the individual clearances for all relevant elimination pathways; for example, the toxicant may be simultaneously metabolized by the liver and excreted by the kidney and at the gills:

$$CL = CL_h + CL_r + CL_b \tag{3.7}$$

where the subscripts refer to hepatic (h), renal (r), and branchial (b) clearance.

Hepatic Clearance

CL_h is limited by the rate of blood flow to the liver. If blood perfusing the liver is completely cleared of chemical, then CL_h is equal to hepatic blood flow. The CL_h may be less than hepatic blood flow, however, if a chemical is poorly metabolized within the liver or if it is reversibly bound to plasma proteins, impeding its transfer from the plasma into hepatocytes. A physiologically based model of hepatic clearance may be developed by assuming that the liver is a well-stirred compartment (Figure 3.18). Toxicant concentrations in blood entering and leaving the liver are denoted as C_a (arterial concentration) and C_v (venous concentration), respectively. Blood flow is represented by Q, and –dX/dt is the rate of toxicant elimination by the liver (the minus sign indicates that chemical is being lost from the system). Metabolizing enzymes may be envisioned as a coating on the walls of the compartment. For this well-stirred model, the rate of elimination of toxicant is:

$$-dX / dt = Q\left(C_a - C_v\right) \tag{3.8}$$

If the right-hand side of this equation is multiplied by C_a/C_a, one obtains:

$$-dX / dt = Q\left(C_a - C_v\right)C_a/C_a \tag{3.9}$$

The ratio $(C_a - C_v)/C_a$ is the hepatic extraction ratio (E). Substituting, the rate of elimination can be written as:

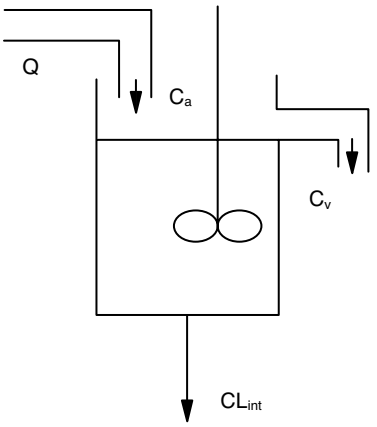

FIGURE 3.18 Well-stirred model of the liver. The liver is represented as a well-stirred volume supplied by a blood flow of Q which contains chemical at a concentration of C_a. The concentration of chemical inside the liver and in blood exiting the liver is C_v, and the activity of metabolizing enzymes is represented by CL_{int}.

$$-dX/dt = QEC_a \tag{3.10}$$

Because $(dX/dt)/C_a$ is equal to CL_h, it is apparent that $CL_h = QE$. Thus, CL_h is that part of the hepatic blood flow that is totally cleared of toxicant.

With this model, the drug concentration in the liver is equal to C_v. Because the rate of elimination is controlled by both Q and by the capacity of hepatic metabolizing enzymes, it is useful to separate these two influences. This can be accomplished by defining the *intrinsic hepatic clearance* (CL_{int}). The CL_{int} is the rate of toxicant elimination divided by the toxicant concentration in the liver; that is, the concentration in contact with metabolizing enzymes:

$$-dX/dt = CL_hC_a = CL_{int}C_v \tag{3.11}$$

and

$$CL_{int} = CL_hC_a/C_v = CL_h/(1-E) \tag{3.12}$$

The rate of chemical elimination by the liver is therefore proportional via the hepatic clearance to the toxicant concentration entering the liver or via the intrinsic hepatic clearance to the toxicant concentration leaving the liver.

A more general model for hepatic clearance that accounts for the possibility of chemical binding to plasma proteins may be written by defining a new reference concentration, the unbound toxicant concentration in the liver ($C_{v,u}$), and a new clearance parameter, the intrinsic unbound hepatic clearance ($CL_{int,u}$). Accounting for plasma binding, the rate of elimination may be written as:

$$-dX/dt = CL_{int,u}C_{v,u} = CL_{int,u}f_uC_v \tag{3.13}$$

where f_u is the fraction of chemical unbound in plasma. $CL_{int,u}$ represents the activity of the metabolizing enzyme toward the toxicant and can be expressed in terms of Michaelis–Menten kinetics as $V_{max}/(K_m + C_{v,u})$. When $C_{v,u}$ is low relative to K_m (< 10%), the relationship simplifies to V_{max}/K_m, and metabolism becomes first order with respect to $C_{v,u}$:

$$-dX/dt = \left(V_{max}/K_m\right)C_{v,u} \tag{3.14}$$

Values for $CL_{int,u}$ and CL_{int} can be calculated from CL_h, Q, and f_u as follows:

$$CL_{int,u} = CL_{int}/f_u \tag{3.15}$$

where

$$CL_{int} = CL_h/(1-E) \tag{3.16}$$

and

$$E = CL_h/Q \tag{3.17}$$

Given values for CL_{int}, f_u, and Q, questions can then be answered about how CL_h, E, and F (oral bioavailability) will change if a change occurs in hepatic blood flow, plasma protein binding, or hepatic metabolism activity. The useful working equations for this model are:

$$E = f_u CL_{int,u}/\left(Q + f_u CL_{int,u}\right) = CL_{int}/\left(Q + CL_{int}\right) \tag{3.18}$$

$$CL_h = Qf_u CL_{int,u}/\left(Q + f_u CL_{int,u}\right) = QCL_{int}/\left(Q + CL_{int}\right) \tag{3.19}$$

The letter F is commonly used to represent the fraction of an oral dose that enters the systemic circulation and has two contributing parts: F_{ab}, which represents the fraction of the dose that is absorbed from the GIT into the blood, and F_{fp}, which represents the fraction of the absorbed dose that escapes elimination by the liver. The overall bioavailability of an orally administered compound is the product of these two fractions:

$$F = F_{ab}F_{fp} \tag{3.20}$$

F_{fp} can also be calculated from CL_{int}, f_u, and Q using the following relationship:

$$F_{fp} = 1 - E = Q/\left(Q + f_u CL_{int,u}\right) = Q/\left(Q + CL_{int}\right) \tag{3.21}$$

The independent variables in Equations 3.18, 3.19, and 3.21 are $CL_{int,u}$, f_u, and Q. The dependent variables are E, CL_h, and F_{fp}. This is an extremely important concept and is reinforced by the diagram shown in Figure 3.19.

The appropriate value to use for Q depends on the reference region. If the reference region is whole blood, then Q should be the whole blood flow to the liver; if plasma, then plasma flow should be used. If the external water is the reference region, then plasma water flow to the liver should be used. In this latter case, if no binding of toxicant occurred in the exposure water, then CL_{int} and $CL_{int,u}$ would have the same value.

The relationships among $CL_{int,u}$, f_u, and Q and the dependent variables E, CL_h, and F_{fp} are simplified when the value of E is small (<0.25) or large (>0.75); for example, for larger E, $CL_{int} >> Q$, and Equation 3.19 becomes $CL_h = Q$. For some toxicants, the value of E lies between 0.25 and 0.75, and the full

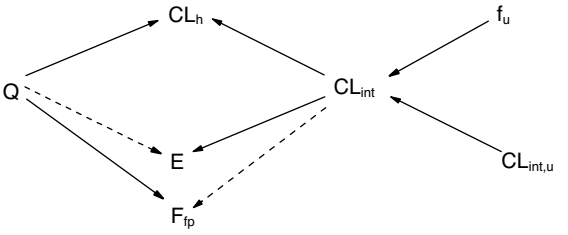

FIGURE 3.19 Relationships between independent physiological and biochemical determinants (Q, $CL_{int,u}$, f_u), and dependent pharmacokinetic parameters (CL_h, E, F_{fp}) of the well-stirred hepatic clearance model: Q, hepatic blood flow; $CL_{int,u}$, intrinsic unbound clearance; f_u, fraction of toxicant unbound in blood; CL_{int}, intrinsic hepatic clearance; CL_h, hepatic clearance; E, hepatic extraction ratio; F_{fp}, fraction of an oral dose that escapes first-pass elimination. Dashed lines depict an inverse relationship between variables; solid lines depict a direct relationship between variables.

equations for E and CL_h have to be used. In such cases, there are no direct proportionalities; $CL_{int,u}$, f_u, and Q all determine E, CL_h, and F_{fp}, and a change in any one of the former variables produces a less than proportional change in the latter.

Renal Clearance

The kidney has two processes by which it clears blood of chemicals: filtration and extraction. Filtration involves the passage of plasma water across the glomerular membrane. Toxicant dissolved in water is transported across the membrane and ends up in the pre-urine. When protein binding occurs, protein-bound compounds remain with the protein in plasma. The plasma concentration of unbound toxicant remains constant during filtration, as both water and toxicant are removed by the filtration process. The equilibrium between bound and free toxicant is therefore not disturbed; consequently, the maximum possible clearance via filtration is the volume flow of plasma water across the glomerular membrane (GFR) multiplied by the fraction of toxicant that is unbound in plasma.

The extraction mechanism only applies when a toxicant is a substrate for an active tubular secretory pathway. Unbound toxicant contained in postglomerular and renal portal blood is transported across the proximal tubule into pre-urine. As in the liver, this may be a high E or low E process, depending on the toxicant. The same extraction model described for the liver can be used for active tubular secretion. In this case, CL_{int} is the activity of the secretory mechanism.

Total clearance by the kidney reflects the net result of filtration, active secretion, and passive reabsorption:

$$CL_r = \text{filtration clearance} + \text{secretion clearance} - \text{reabsorption}$$

Passive reabsorption is expected to be minimal in freshwater fish because the kidney does not reabsorb much water from urine and thereby concentrate the toxicant. In marine fish, the kidney conserves water by reabsorption and a greater potential exists for toxicant reabsorption from urine.

Branchial Clearance

Fish gills are an important site for toxicant uptake and elimination, and both processes can be described using clearance concepts. Unlike hepatic and renal clearance, however, the mathematical description of branchial clearance must be bidirectional to reflect the fact that chemical flux occurs in both directions. Depending on the concentration gradient across the gills, fish may clear chemical from inspired water or from blood flowing through the gills. The principal limitations on chemical uptake at fish gills were identified by Hayton and Barron (1990). If the permeability of the gill epithelium to a toxicant is low, diffusion can limit exchange, and branchial clearance is controlled by the product of gill permeability (K_d) and the surface area for diffusion (A):

$$CL_b = K_dA \tag{3.22}$$

The permeability coefficient is proportional to chemical diffusivity in the gill epithelium (D) and is inversely related to the effective thickness of the diffusion barrier (h):

$$K_d = D/h \tag{3.23}$$

If diffusive flux is limited by chemical diffusion within nonaqueous portions of the gill epithelium, it is appropriate to incorporate a membrane–water partition coefficient (P_{mw}), which would be expected to correlate positively with chemical lipophilicity:

$$K_d = DP_{mw}/h \tag{3.24}$$

The resulting permeability coefficient is identical to that given previously in Equation 3.1. Alternatively, if chemical diffusion in the aqueous phase of the membrane limits flux, Equation 3.23 is appropriate. Similarly, the value of D applies to the phase that constitutes the principal barrier to diffusive flux.

Chemical diffusivity in aqueous solution may be calculated from equations given in standard physical chemistry texts and is expected to decline with increasing molecular volume.

If the permeability of the gill epithelium to a toxicant is sufficiently high, branchial uptake may be limited by the rate of blood flow through the gill lamellae. Chemical binding to plasma proteins or to cells in the blood would increase the capacity of blood to carry toxicant away from the gills. The uptake clearance when blood flow is the rate-limiting factor may be expressed as:

$$CL_b = Q_c P_{bw} \tag{3.25}$$

where Q_c is the total cardiac output, and P_{bw} is an equilibrium blood–water partition coefficient.

Because the value of P_{bw} can be very large, it is possible for water flow across the absorbing surface of the gills to control the rate of chemical uptake. In this case, the value of CL_b is equal to the effective respiratory volume (Q_w), which is the flow rate of inspired water that exchanges with blood:

$$CL_b = Q_w \tag{3.26}$$

To a first approximation, the potential rate limitations on branchial flux can treated as if they were resistances in series in an electrical circuit (Hayton and Barron, 1990):

$$CL_b = \left(h/DP_{mw}A + 1/Q_c P_{bw} + 1/Q_w\right)^{-1} \tag{3.27}$$

A more complex model based on the counter-current structure of fish gills was developed by Erickson and McKim (1990a,b) and is described below in the section on physiologically based toxicokinetic models; however, in the case where one resistance is the principal determinant of chemical flux (by virtue of being much greater than the other two), the two models reduce to a common description.

Compartmental Models for Fish

The use of compartmental models to characterize the kinetics of absorption, distribution, and elimination of exogenous and endogenous substances by a variety of species is well established. The foundations of this approach (Wagner, 1981) were developed by Haggard (1924), Widmark and Tandberg (1924), Dominquez and Pomerene (1934), and Teorell (1937). In the decades since this early work was published, there have been refinements in modeling concepts and in the techniques for data analysis. A number of textbooks on this topic are available (Gibaldi and Perrier, 1982; Rowland and Tozer, 1995; Wagner, 1993; Welling, 1986). Riggs (1963) defined the term *compartment* as follows:

> If a substance, S, is present in a biological system in several distinguishable forms or locations, and if S passes from one form or location to another form or location at a measurable rate, then each form or location constitutes a separate compartment for S.

In the context of toxicokinetics, a parent toxicant and its metabolites would be forms of substance S, and a location would be one or more tissues in which the forms had similar kinetic behavior. As an example, a tank of exposure water might be a compartment from which toxicant absorption occurs, the blood and highly perfused tissues might constitute a second compartment, the poorly perfused tissues would be a third compartment, and a metabolite might require specification of a fourth compartment (Figure 3.20A).

Compartments are assumed to behave like a tank that contains a well-stirred fluid; that is, toxicant that enters a compartment is assumed to distribute instantaneously and linearly, such that the concentration is everywhere proportional to the amount of chemical in the compartment. The number of compartments is generally small (1 to 5) to maintain mathematical simplicity and the capability to fit model-based equations to experimental data. Body compartments are usually arranged so chemical enters and exits from only one of the compartments, the so-called central compartment. With multiple-compartment models, the added peripheral compartments are usually attached to the central compartment so chemical exchanges between the central and each peripheral compartment; this configuration is termed *mammillary*, as opposed to the *catenary* configuration, which refers to a chain-like series of compartments.

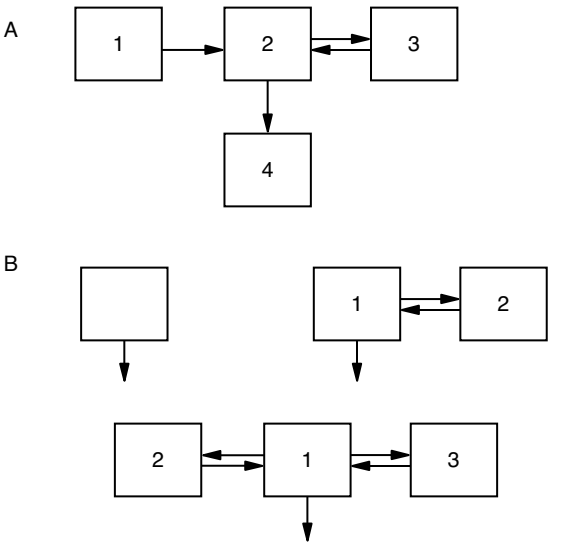

FIGURE 3.20 Examples of compartmental models. (A) A model appropriate for toxicant absorbed from the gastrointestinal tract (GIT) and eliminated by formation of a metabolite. Compartment 1, GIT; compartments 2 and 3, the body lumped into rapidly and slowly equilibrating tissues; compartment 4, the metabolite. Arrows represent first-order kinetic processes. (B) Mammillary models that represent the body as one, two, or three well-stirred compartments.

Typical mammillary configurations are shown in Figure 3.20B. Some considerations in the selection of the number of compartments include the purpose of the model, the frequency with which toxicant concentration is measured, and the number of sites from which samples are taken for determination of toxicant and metabolite concentrations.

Volume of Distribution

The size of a compartment is characterized by an apparent volume of distribution, V, that has units of volume or volume normalized to body size (weight or surface area). The apparent volume of distribution of the central compartment (compartment 1 in Figure 3.20B) is the amount of toxicant in the compartment divided by the toxicant concentration in a reference region. The reference region is the fluid or tissue in which the toxicant concentration is measured, commonly blood or plasma. In the case of plasma, for example, the value of V_1 would represent the volume of plasma that would be required to account for all toxicant in the central compartment. In studies with small fish, the exposure water is commonly used as the reference region. In this case, the value of V_1 represents the volume of water that would be required to account for all of toxicant in the central compartment under equilibrium conditions and in the absence of clearance from the central compartment. Under these conditions, the concentration in the exposure water equals the concentration of toxicant freely dissolved in plasma water.

The apparent volumes of distribution of the peripheral compartments are based on the same reference region as that of the central compartment, and the summed volume of all compartments is the apparent steady-state volume of distribution (V_{ss}). The magnitude of the V_{ss} is determined by the affinity of a toxicant for the reference region relative to that for other tissues and fluids. The fractional water content of most fish tissues is about 65 to 80% (Bertelsen et al., 1998). If plasma were used as the reference region, a V_{ss} of about 0.65 to 0.8 L/kg would be expected for a toxicant that distributed only into body water and did not bind to plasma proteins. Alternatively, if the toxicant reversibly bound to plasma proteins but had relatively little affinity for other tissues, the V_{ss} would be less than 0.65 L/kg. Conversely, if the toxicant had high affinity for one or more tissues (e.g., high lipid solubility resulting in high concentrations in fat), the V_{ss} would be much greater than 1 L/kg. A physiologically based model of apparent volume of distribution is described in Rowland and Tozer (1995). V_{ss} values measured in fish vary widely (Table 3.2).

TABLE 3.2

Apparent Volumes of Distribution (V_{ss}) for Selected Chemicals in Fish

Chemical	Temperature (°C)	Species	Reference Region[a]	V_{ss} (L/kg)	Ref.
Nitrofurantoin	25	Channel catfish	Plasma	0.08	Stehly and Plakas (1993)
Sulfachlorpyridazine	22	Channel catfish	Plasma	0.34[b]	Alavi et al. (1993)
Diethylhexylphthalate	12	Rainbow trout	Plasma	0.35	Barron et al. (1987b)
Sulfadimethoxine	10	Atlantic salmon	Plasma	0.39	Samuelsen et al. (1995)
Sulfadimethoxine	13	Rainbow trout	Plasma	0.42	Kleinow et al. (1992)
Sulfadiazine	24	Carp	Plasma	0.60[c]	Nouws et al. (1993)
Nalidixic acid	14	Rainbow trout	Plasma	0.96	Jarboe et al. (1993)
Benzocaine	12	Rainbow trout	Plasma	1.0	Ma (2000)
Paraoxon	12	Rainbow trout	Plasma	1.1	Abbas et al. (1996)
Parathion	12	Rainbow trout	Plasma	1.3	Abbas et al. (1996)
Chlorpyrifos	22	Channel catfish	Blood	1.5	Barron et al. (1991)
Sarafloxacin	10	Atlantic salmon	Plasma	2.3	Martinsen and Horsberg (1995)
Ormetoprim	10	Atlantic salmon	Plasma	2.5	Samuelsen et al. (1995)
Diquat	25	Channel catfish	Plasma	2.9	Schultz et al. (1995)
Oxolinic acid	5	Atlantic salmon	Plasma	2.9[c]	Rogstad et al. (1993)
Flumequine	5	Atlantic salmon	Plasma	3.1[c]	Rogstad et al. (1993)
Flumequine	10	Atlantic salmon	Plasma	3.5	Martinsen and Horsberg (1995)
Trimethoprim	24	Carp	Plasma	4.0[c]	Nouws et al. (1993)
Oxolinic acid	10	Atlantic salmon	Plasma	5.4	Martinsen and Horsberg (1995)
Trifluralin	12	Rainbow trout	Plasma	5.7	Schultz and Hayton (1993)
Trimethoprim	10	Rainbow trout	Plasma	6.0	Tan and Wall (1995)
Enrofloxacin	10	Atlantic salmon	Plasma	6.1	Martinsen and Horsberg (1995)
Methyltestosterone	15	Rainbow trout	Plasma	6.1	Vick and Hayton (2001)
Proflavine	12	Rainbow trout	Plasma	28	Yu (1996)
Acriflavine	12	Rainbow trout	Plasma	31	Yu (1996)
Aminoantipyrine	20	Goldfish	Water	0.55	Kaka and Hayton (1978)
Sulfapyridine	20	Goldfish	Water	0.59	Lo and Hayton (1981)
Ethanol	20	Goldfish	Water	0.68	Kaka and Hayton (1978)
Pentachlorophenol	12	Rainbow trout	Water	478	Stehly and Hayton (1989a)
Trifluralin	12	Rainbow trout	Water	3200	Schultz and Hayton (1993)
Diethylhexylphthalate	23	Sheepshead minnow	Water	19,000	Karara and Hayton (1984)

[a] V_{ss} values referenced to plasma and blood were determined by collecting serial samples after intravascular administration of the chemical. Values referenced to water were determined from whole-body concentrations during waterborne exposures.

[b] V_{ss} was calculated using noncompartmental analysis as the product of mean residence time (MRT) and total body clearance (CL).

[c] β-Phase volume of distribution (V_β).

One-Compartment Model

Intravascular Administration

The simplest compartment model assumes that the fish body behaves like a single, well-stirred compartment. In this section, the one-compartment model is developed for a toxicant that is administered by intravascular injection. Intravascular administration is usually accomplished by injecting a solution of the toxicant via an indwelling catheter placed in the dorsal aorta. The dose may be administered as a rapid injection (bolus) or as a constant-rate infusion using an infusion pump. After the dose is administered, samples of plasma are removed at various times to measure the toxicant concentration (C_p).

Bolus Dose—For a toxicant administered as a bolus dose, its rate of elimination (dX/dt; mass/time) is:

$$-dX/dt = CLC_p \tag{3.28}$$

Division of both sides by V, followed by integration, yields an equation that predicts the time course of C_p:

$$C_p = C_0 e^{-(CL/V)t} \tag{3.29}$$

where t is time and C_0 is the plasma concentration at t = 0 (i.e., the dose/V). This equation predicts that the plasma concentration will decline exponentially and a graph of log C_p vs. time should give a straight line. From the slope of the line, the ratio CL/V may be obtained, which is commonly referred to as the elimination rate constant (k_{el}):

$$k_{el} = -2.3 \cdot \text{slope} = CL/V \tag{3.30}$$

The elimination half-life ($t_{1/2}$) is calculated from k_{el}:

$$t_{1/2} = 0.693/k_{el} = 0.693V / CL \tag{3.31}$$

This relationship shows that a change in $t_{1/2}$ may result from a change in CL or V. Barron et al. (1987b) found that the $t_{1/2}$ for di-2-ethylhexylphthalate (DEHP) in rainbow trout increased with acclimation temperature and that this increase was primarily due to changes in V and not CL. The *y*-axis intercept of the semilog plot gives an estimate of C_0 that is used to calculate a value for V:

$$V = \text{dose}/C_0 \tag{3.32}$$

As a minimum, the number of plasma samples should be three times the number of parameters to be estimated (in this case, six samples to estimate the two parameters CL and V). These samples should be uniformly spaced and should be taken over a time span at least three times the $t_{1/2}$.

Infusion Dose—In some cases, it may be advantageous to administer the dose at a constant rate (R_0). This approach avoids the high transient plasma concentration associated with a bolus dose and provides a larger volume of solvent to dissolve the toxicant. The plasma concentration will increase exponentially during the infusion, followed by an exponential decline after the infusion is stopped. During the infusion, the predicted plasma concentration is:

$$C_p = \left(R_0/CL\right)\left(1 - e^{-k_{el}t}\right) \tag{3.33}$$

After three to four half-lives, the exponential approaches a value of zero, and a steady-state condition is achieved in which the rate of infusion equals the rate of elimination of the toxicant.

Plasma samples are generally obtained after the infusion has been stopped using the same cannula employed to infuse the toxicant. The value of k_{el} is obtained from the slope of a semilog plot of the post-infusion plasma data (Equation 3.30). If the infusion was sufficient to achieve a steady-state condition, CL can be calculated from:

$$CL = R_0/C_{ss} \tag{3.34}$$

where C_{ss} is the steady-state plasma concentration, estimated from the *y*-axis intercept of a semilog plot of the post-infusion C_p,t profile. The volume of distribution (V) can then be calculated as:

$$V = CL/k_{el} \tag{3.35}$$

If the infusion was stopped prior to steady state, k_{el} may still be estimated from the slope of the semilog plot, but estimation of CL and V is less straightforward because the amount of toxicant in the fish at the end of the infusion is unknown. Equation 3.33 may be rearranged to give the following expression for estimation of CL, which can then be used along with k_{el} to estimate V:

$$CL = \left(R_0/C_{end}\right)\left(1 - e^{-k_{el}t_{inf}}\right) \tag{3.36}$$

where C_{end} is the concentration in plasma at the end of the infusion, estimated from the semilog plot, and t_{inf} is the duration of the infusion.

Waterborne Exposure

Kinetic studies with fish may be conducted by exposing small fish or juveniles of larger species to a chemical in water. Typically, the exposure is initiated with a fixed number of fish and at each sampling time a subset of animals is collected to determine whole-body chemical concentrations. The exposure may be conducted using a *flow-through* system that maintains a constant concentration of toxicant in the exposure water. Alternatively, fish may be exposed in a static system, which may or may not be renewed during the progress of a test. The aqueous concentration of toxicant in a static exposure system may decline over time due to absorption by the test organisms. Additional losses may occur due to adsorption to the test container, volatilization, photodegradation, and microbial metabolism.

Flow-Through Exposure—The rate of toxicant accumulation in fish dosed in a flow-through exposure (dX/dt; usually normalized to body weight) is proportional to the difference between the concentration of toxicant in the exposure water (C_w) and the concentration unbound in the plasma water ($C_{p,u}$):

$$dX / dt = \rho\left(C_w - C_{p,u}\right) \tag{3.37}$$

The C_w is constant during the exposure, and the $C_{p,u}$ can be replaced with X/V, where V is the apparent volume of distribution of the toxicant referenced to the exposure water. The units of V and ρ are usually normalized to body weight; for example, if X had units of ng/g, and C_w and $C_{p,u}$ had units of ng/mL, then V would have units of mL/g and ρ would have units of mL/hr/g. ρ is often the same as CL_b, as discussed on pages 95 to 96. Integration of Equation 3.37 gives an equation for X as a function of time:

$$X = VC_w\left(1 - e^{-(\rho/V)t}\right) \tag{3.38}$$

This equation can be fit to experimentally determined values of X at various times to determine values for ρ and V, and the elimination half-life can be calculated as:

$$t_{1/2} = 0.693V/\rho \tag{3.39}$$

Equation 3.38 predicts that X will exponentially approach a limiting value equal to VC_w. The value of V is equivalent to the bioconcentration factor (BCF), as described later in this chapter.

When a metabolite of the toxicant is formed during the exposure, this model may be extended to permit simultaneous characterization of its toxicokinetics. One approach is to determine the total amount of metabolite formed, including that in the fish and in the exposure water. Quantification of the amount eliminated to the exposure water is complicated by the need to measure metabolite in water flowing from the exposure vessel. The appropriate rate equations for this situation are:

$$dX / dt = \rho\left(C_w - C_{p,u}\right) - CL_m C_{p,u} \tag{3.40}$$

$$dM / dt = CL_m C_{p,u} \tag{3.41}$$

where CL_m is the metabolism clearance for metabolite formation. The integrated form of Equation 3.40 is (Benet, 1972):

$$X = X_{ss}\left(1 - e^{-k_{el}t}\right) \tag{3.42}$$

where

$$X_{ss} = \rho VC_w / \left(\rho + CL_m\right) \tag{3.43}$$

$$k_{el} = \left(\rho + CL_m / V\right) \tag{3.44}$$

The integrated form of Equation 3.41 is:

$$M = \rho CL_m C_w t / (\rho + CL_m) - CL_m X_{ss} (1 - e^{-k_{el} t}) / (\rho + CL_m) \quad (3.45)$$

The dependent parameters ρ, V, and CL_m, can be estimated by fitting Equations 3.42 and 3.45 to measured values of X and M. Initial parameter estimates (required as inputs to the data-fitting software) can be obtained from a graph of (X_{ss} – X) vs. time on semilogarithmic coordinates. The slope of this plot is equal to k_{el}/–2.3. The value of ρ can then be estimated from the relationship:

$$\rho = k_{el} X_{ss} / C_w \quad (3.46)$$

Importantly, the presence of a metabolic elimination pathway cannot be deduced from the X,t profile alone. The model-predicted M,t profile (Equation 3.45) indicates that M will increase linearly with time after a transient period (T_{lag}) that is determined by the half-life of the parent toxicant:

$$t_{1/2} = 0.693V(\rho + CL_m) = 0.693/k_{el} \quad (3.47)$$

By extrapolating the linear part of the M,t plot to the time axis, it is possible to obtain an estimate of T_{lag}, which can be shown to be equal to $1/k_{el}$ by solving Equation 3.45 when M = 0. Substituting, Equation 3.45 may now be written as:

$$M = [\rho CL_m C_w / (\rho + CL_m)][t - T_{lag}(1 - e^{-t/T_{lag}})] \quad (3.48)$$

The BCF for this case is:

$$BCF = X_{ss}/C_w = \rho V / (\rho + CL_m) \quad (3.49)$$

Rather than being equivalent to V (as in the model without metabolism), the BCF is now a fraction of V that is equal to the ratio $\rho/(\rho + CL_m)$ (Karara and Hayton, 1984).

Static Exposure—In a static exposure, the C_w may decline due to uptake of chemical by the exposed organisms. Under these circumstances, Equation 3.37 may be integrated to give:

$$X = \frac{VV_w C_{w,0}}{V_w + V}\left[1 - e^{-((1/V_w)+(1/V))\rho t}\right] \quad (3.50)$$

where $C_{w,0}$ is the initial concentration of chemical in the exposure water, and V_w is the volume of exposure water. The water concentration would decline according to the relationship:

$$-dC_w/dt = \rho(C_w - C_{p,u})/V_w \quad (3.51)$$

Integrating, one obtains:

$$C_w = C_{w,0}\left[1 - \frac{V}{V_w + V}\left(1 - e^{-((1/V_w)+(1/V))\rho t}\right)\right] \quad (3.52)$$

An examination of Equation 3.50 suggests that the time to achieve steady state is influenced by the value of V_w and that it is shorter than it would have been had C_w remained constant (as in a flow-through exposure). This influence of V_w on the time to steady state can be exploited to shorten the time required to characterize the X,t profile when the time to steady state would otherwise be very long. By making V_w relatively small compared with V, the time to steady state is minimized.

Body Weight—A modification of the static exposure model is required when experiments are conducted using individual fish of different sizes. Larger fish will deplete the exposure solution faster, but the amount of toxicant absorbed per gram of body weight will be less than in smaller fish because the absorbed toxicant distributes into a larger body size. In this case, Equations 3.32 and 3.51 must be rewritten to explicitly consider fish body weight (W):

$$dX/dt = \rho W\left(C_w - C_{p,u}\right) \tag{3.53}$$

$$-dC_w/dt = \rho W\left(C_w - C_{p,u}\right)/V_w \tag{3.54}$$

Integrating, one obtains equations analogous to those given previously:

$$X = \frac{VV_wC_{w,0}}{WV_w + V}\left[1 - e^{-\left((W/V_w)+(1/V)\right)\rho t}\right] \tag{3.55}$$

$$C_w = C_{w,0}\left[1 - \frac{V}{WV_w + V}\left(1 - e^{-\left((W/V_w)+(1/V)\right)\rho t}\right)\right] \tag{3.56}$$

To fit Equation 3.55 and Equation 3.56 to experimental data, both time and fish weight are entered as independent variables, along with measured values of C_w and X.

Oral Administration

A single oral dose may be administered to a fish that has been prepared with an indwelling vascular catheter. If the absorption kinetics are first order and monoexponential, then the rate of appearance of chemical in the fish is:

$$dX/dt = k_{ab}X_g - CLC_p \tag{3.57}$$

where k_{ab} is the absorption rate constant, and X_g is the amount of toxicant remaining in the GIT. From this equation it is possible to derive the following relationship:

$$C_p = k_{ab}F \cdot dose\left(e^{-(CL/V)t} - e^{-k_{ab}t}\right)/V\left(k_{ab} - (CL/V)\right) \tag{3.58}$$

where F is the fraction of the dose that reaches the systemic circulation of the fish (oral bioavailability; see earlier section on hepatic clearance). The value of F may be estimated by comparison of the area under the C_p,t profile with the area determined after an intravascularly administered dose, where F is unity.

When the dietary exposure is continuous, the rate of change of the amount of chemical in the fish is:

$$dX/dt = FR_{in} - CLC_p \tag{3.59}$$

where R_{in} is the rate of chemical ingestion. In this case, the mass of chemical exponentially approaches a constant or steady-state value:

$$X_{ss} = \left(FR_{in}V/CL\right)\left(1 - e^{-k_{el}t}\right) \tag{3.60}$$

Two-Compartment Model

A one-compartment model assumes that a chemical distributes instantaneously throughout all tissues of the exposed animal. This assumption often fails because chemical distribution to each tissue is linked to blood perfusion rate, and tissue-specific perfusion rates vary considerably. When the kinetics of internal distribution affect the shape of the plasma concentration–time profile, a higher order compartment model is required. Perhaps the most common example of this phenomenon occurs when log-transformed plasma concentration data from an intravascular dosing study decrease in a *biphasic* manner (Figure 3.21). The

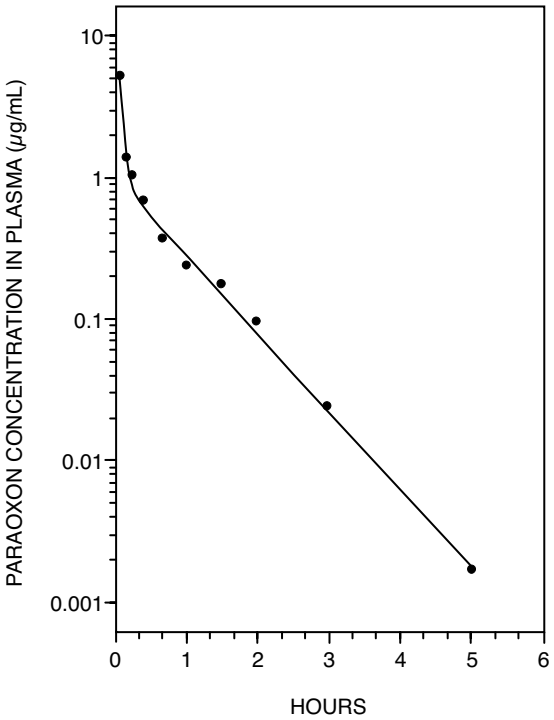

FIGURE 3.21 Paraoxon plasma concentration–time profile in rainbow trout after intraarterial injection. Symbols represent the experimentally determined concentrations, and the line represents the least-squares fit of a two-compartment-model-based biexponential equation. (Adapted from Abbas, R. et al., *Toxicol. Appl. Pharmacol.*, 136, 194–199, 1996.)

early and often rapid decline in plasma concentration is thought to be due primarily to a redistribution of the injected compound, while the later elimination or β phase is controlled by rate limitations on chemical elimination. A two-compartment model for intravascular administration of a bolus dose is described in the following section. Space does not permit the presentation of other multiple-compartment models; however, the principles illustrated by this relatively simple model apply generally to more complex models.

Intravascular Bolus Administration

This widely used model represents the body as two compartments (Figure 3.20B). Toxicant enters the central compartment, distributing instantaneously. Some of this toxicant moves from the central compartment into a peripheral compartment. Once it is in the peripheral compartment, the toxicant is again assumed to distribute instantaneously. The central compartment generally represents the blood and highly perfused tissues such as the liver, kidney, and GIT. The peripheral compartment usually represents poorly perfused tissues, including muscle and fat. The brain may be associated with either compartment, depending on whether the blood–brain barrier limits the rate of toxicant exchange between the blood and brain tissues.

Following the intravascular administration of a bolus dose (X_0), mass-balance equations that describe the rate of change of the amount of toxicant in compartments one and two may be written as follows:

$$dX_1/dt = k_{21}X_2 - k_{10}X_1 - k_{12}X_1 \tag{3.61}$$

$$dX_2/dt = k_{12}X_1 - k_{21}X_2 \tag{3.62}$$

where X_1 and X_2 are the amounts of toxicant in compartments 1 and 2, k_{12} and k_{21} are first-order intercompartmental transfer rate constants, and k_{10} is a first-order rate constant for chemical elimination

from the central compartment. These equations may then be integrated to give X_1 and X_2 as functions of time:

$$X_1 = \left[X_0/(\alpha-\beta)\right]\left[(k_{21}-\beta)e^{-\beta t} - (k_{21}-\alpha)e^{-\alpha t}\right] \tag{3.63}$$

$$X_2 = \left[X_0 k_{12}/(\alpha-\beta)\right]\left(e^{-\beta t} - e^{-\alpha t}\right) \tag{3.64}$$

where α and β are rate constants that are comprised of the model rate constants:

$$\alpha = 0.5\left\{(k_{12}+k_{21}+k_{10}) + \left[(k_{12}+k_{21}+k_{10})^2 - 4k_{21}k_{10}\right]^{1/2}\right\} \tag{3.65}$$

$$\beta = 0.5\left\{(k_{12}+k_{21}+k_{10}) - \left[(k_{12}+k_{21}+k_{10})^2 - 4k_{21}k_{10}\right]^{1/2}\right\} \tag{3.66}$$

The total amount of toxicant in the body ($X = X_1 + X_2$) is:

$$X = \left[X_0/(\alpha-\beta)\right]\left[(k_{12}+k_{21}-\beta)e^{-\beta t} - (k_{12}+k_{21}-\alpha)e^{-\alpha t}\right] \tag{3.67}$$

The plasma concentration is X_1/V_1, or:

$$C_p = \left[X_0/V_1(\alpha-\beta)\right]\left[(k_{21}-\beta)e^{-\beta t} - (k_{21}-\alpha)e^{-\alpha t}\right] \tag{3.68}$$

which is an equation of the form:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \tag{3.69}$$

This equation can be fit to the C_p,t profile by graphical or nonlinear least-squares methods to obtain estimates for A, α, B, and β. These values may then be used to calculate model parameters and other parameters of interest (Gibaldi and Perrier, 1982):

$$V_1 = X_0/(A+B) \tag{3.70}$$

$$k_{21} = (A_\alpha + B_\beta)/(A+B) \tag{3.71}$$

$$k_{10} = \alpha\beta/k_{21} \tag{3.72}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{10} \tag{3.73}$$

$$V_2 = V_1(k_{12}/k_{21}) \tag{3.74}$$

$$V_{ss} = V_1 + V_2 \tag{3.75}$$

$$V_\beta = V_1(k_{12}+k_{21}-\beta)/(k_{21}-\beta) = V_1 k_{10}/\beta \tag{3.76}$$

$$CL = k_{10}V_1 = \beta V_\beta \tag{3.77}$$

$$t_{1/2,\alpha} = \ln 2/\alpha \tag{3.78}$$

$$t_{1/2,\beta} = \ln 2/\beta \tag{3.79}$$

V_{ss} and V_β are the steady-state and β-phase volumes of distribution. The former, when multiplied by C_p at steady state (e.g., in a continuous waterborne exposure), gives the amount of toxicant in the organism.

The latter, when multiplied by C_p during the log-linear or β phase, gives the amount of toxicant in the organism. V_{ss} reflects only distribution, whereas V_β is, in addition, a function of the kinetics of distribution and elimination. $V_\beta \geq V_{ss}$ because during the β phase there is a positive chemical gradient from compartment 2 to compartment 1. For a given plasma concentration, this results in more toxicant in the organism during the β phase than during steady state. The $t_{1/2,\alpha}$ term is characteristic of the time required for distribution, and $t_{1/2,\beta}$ is the elimination half-life of the toxicant, which is similar to the half-life for a one-compartment model.

The CL parameter represents the total body clearance and may reflect elimination by several pathways (e.g., branchial elimination, renal excretion, and hepatic metabolism; see Equation 3.7). The amount of chemical eliminated by each pathway is directly proportional to the magnitude of the clearance for the pathway. To obtain a clearance value for each pathway, the amounts eliminated by each pathway must be determined. The individual clearances may then be calculated as follows:

$$CL_h = \left(M_\infty / X_0\right) CL \tag{3.80}$$

$$CL_r = \left(X_{r,\infty} / X_0\right) CL \tag{3.81}$$

$$CL_b = \left(X_{b,\infty} / X_0\right) CL \tag{3.82}$$

where M_∞ is the total amount of metabolite formed, and $X_{r,\infty}$ and $X_{b,\infty}$ are the amounts of chemical eliminated in urine and expired branchial water. Calculation of $X_{r,\infty}$ requires the use of urinary duct catheters to quantitatively collect the urine (Kleinow, 1991).

Utility of Compartmental Models for Fish

Compartmental models have the advantage of requiring limited data relative to physiologically based models and require only time-series data for the parent compound in a reference tissue, such as plasma, to infer chemical dynamics in other tissues and the whole fish (Barron et al., 1990). Compartmental modeling allows estimation of the key pharmacokinetic parameters characterizing chemical uptake from water (CL_b), distribution (V), and persistence (half-life). The modeled compartments generally do not have any physiological or anatomical reality, but clearance-based constants do allow a degree of physiological interpretation and realism. A common inference is that after intravascular administration of a chemical, the lower limit on the size of V is the blood or plasma volume, and a larger V indicates that the chemical is distributing outside of the vascular system. The magnitude of CL_b is directly interpretable as a proportion of ventilation volume and cannot exceed ventilation volume unless biotransformation occurs in the blood. Compartmental models are limited in their ability to extrapolate across species but have been used to assess the effects of body size and environmental factors such as temperature on toxicokinetics in fish and to discern presystemic biotransformation in the gill (Barron et al., 1987b, 1989; Schultz and Hayton, 1994).

Physiologically Based Toxicokinetic Models

Physiologically based toxicokinetic (PBTK) models are founded on the premise that chemical uptake and disposition can be described from anatomical, physiological, and biochemical attributes of the exposed organism and physicochemical characteristics of the compound. The compartments in a physiological model correspond to actual tissues and organs, and chemical transport within the animal is defined by blood flow relationships. This mechanistic foundation provides for the possibility of extrapolating kinetic information among species and chemicals by making appropriate adjustments of biological and chemical inputs.

Mass-balance differential equations that describe chemical kinetics in a tissue exchanging with blood were developed in the 1930s (Teorell, 1937). The computing power required to simultaneously solve equations for several tissues was not widely available, however, until the 1960s. Early use of the physiological modeling approach consisted primarily of describing the kinetics of chemotherapeutic

agents (Gerlowski and Jain, 1983); subsequently, this approach was adopted by toxicologists interested in extrapolating data from laboratory test animals to humans. Much of the early work in this field was focused on modeling volatile organic compounds (Andersen, 1981; Ramsey and Andersen, 1984). Additional models have been developed for a variety of nonvolatile toxicants, including several metals (Medinsky and Klaassen, 1996). Increasingly, PBTK models are being linked with biologically based dose–response (BBDR) models to provide a complete (toxicokinetic and toxicodynamic) toxicological description (Connolly et al., 1988).

One early application of the PBTK modeling approach was to compare chemical distribution and elimination in fish and rodents. Models for methotrexate in stingrays (Zaharko et al., 1972) and phenol red in the dogfish shark (Bungay et al., 1976) were designed to simulate experiments in which compounds were injected intravascularly, and elimination was limited to urinary and fecal routes. The first PBTK model for fish that incorporated an environmentally relevant route of exposure (branchial) was developed by Nichols et al. (1990). Later models have incorporated both dermal and dietary routes of exposure (Nichols et al., 1996, 1998, 2004a). In this section, we review the principles of PBTK modeling with fish. Mathematical details pertaining to mass-balance tissue descriptions are deemphasized, as this information is available in several reviews (Gerlowski and Jain, 1983; Krishnan and Andersen, 2001; Rowland, 1985). This section focuses instead on route of exposure considerations as they relate to fish. An emphasis is placed on factors that distinguish fish and mammalian models, either in terms of model structure or the ways in which the models are used. In several instances, empirical data are reviewed to define the chemical kinetic behavior that successful models must simulate.

Model Structure

A PBTK model consists of several tissue compartments, connected in a manner that is consistent with the cardiovascular system of the exposed organism (Figure 3.22). Additional details follow from the type of exposure, characteristics of the test compound, and goals of the modeling exercise. Often, it is necessary to ignore known or suspected complexity to simplify the task of parameter estimation. In general, a modeler strives to create the simplest possible structure that will satisfactorily represent the behavior of the system under study. The number and identity of tissue compartments can vary greatly. An emphasis is usually placed on tissues that control the kinetics of uptake and elimination, as well as those that are important toxicologically.

Physiological and anatomical attributes of fish that influence chemical uptake and disposition were reviewed earlier in this chapter. Several attributes are singled out here because they tend to distinguish fish and mammalian PBTK models. First, chemical uptake by inhalation exposure can be adequately simulated in mammals by modeling the lungs as a mixed chemical reactor. Chemicals taken up across the lung travel first to the heart and then to the rest of the body. In contrast, the gills of many fish species are more appropriately modeled as a counter-current exchange system, and chemicals taken up across the gills travel throughout the body before reaching the heart. Second, the kidney in many fish is supplied by venous blood draining both the trunk musculature and skin. Although this portal blood does not appear to be available for glomerular filtration, it may contribute to urinary elimination of compounds that are actively secreted in the proximal tubule. Because fish are poikilothermic, physiological and metabolic parameters such as ventilation volume and cardiac output can vary greatly with ambient temperature, thereby having a large impact on chemical kinetics. Finally, it is important to recognize that important physiological differences exist among fish species because of differences in life history and reproductive strategy, dietary preferences, and the need to adapt to different environmental conditions. Incorporation of these differences into any given model depends on whether they influence the kinetics of the chemical.

The compartments in a PBTK model correspond to tissues and organs with similar kinetic characteristics. For a well-mixed compartment that receives only arterial blood, a mass-balance equation may be written as:

$$dX_i/dt = Q_i\left(C_a - C_{vi}\right) \tag{3.83}$$

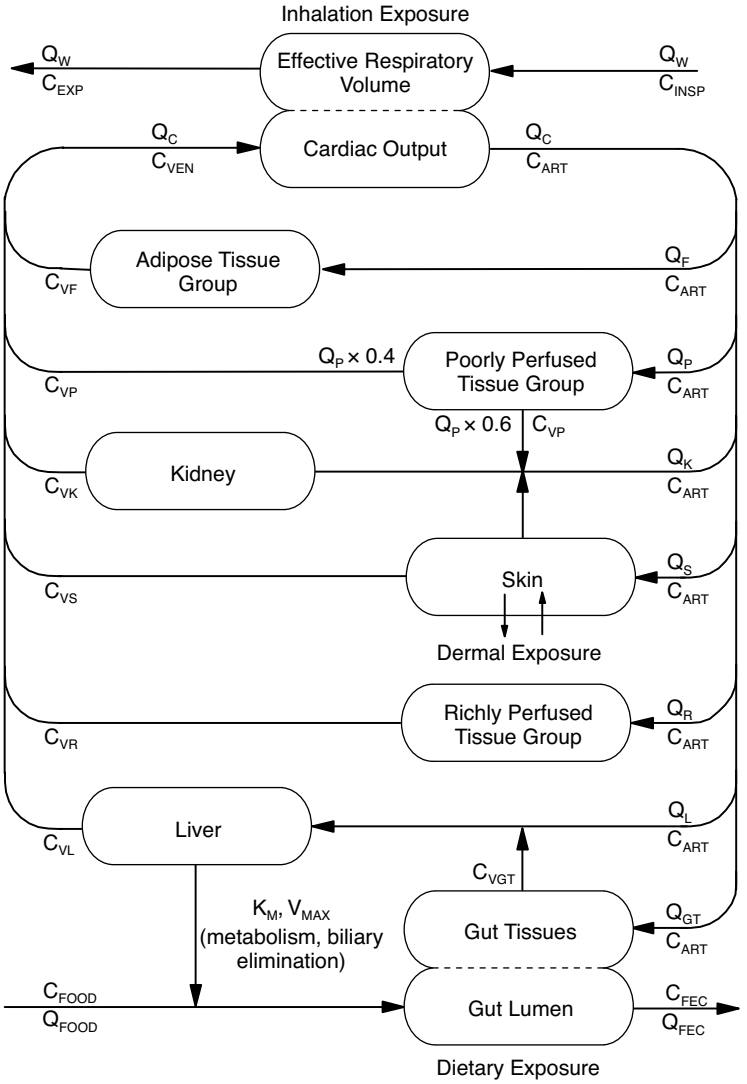

FIGURE 3.22 Schematic representation of a physiologically based toxicokinetic model for fish. Q_F, Q_P, Q_K, Q_S, Q_R, Q_L, and Q_{GT} refer to adipose tissue, poorly perfused tissue (white muscle, bone, and connective tissue), kidney, skin, richly perfused tissue (stomach, upper intestinal tract, spleen, and gonads), liver, and lower intestine blood flows as proportions of Q_C, the cardiac output. Q_W is the flow rate of inspired water that is available to exchange with blood at the gills, and C_{INSP} is the chemical concentration in this water. Q_{FOOD} and Q_{FEC} are the bulk flow rates of food and feces. C_{FOOD} and C_{FEC} are the chemical concentrations in food and feces. C_{VF}, C_{VP}, C_{VK}, C_{VS}, C_{VR}, C_{VL}, and C_{VGT} refer to chemical concentrations in venous blood draining these tissues, and C_{VEN} is the chemical concentration in mixed venous blood. K_M and V_{MAX} are kinetic rate and capacity parameters for saturable metabolism in the liver.

where X_i is the amount of chemical in the compartment, Q_i is the arterial blood flow rate, C_a is the chemical concentration in arterial blood and C_{vi} is the chemical concentration in venous blood draining the compartment. By definition, the rate of chemical accumulation by a tissue cannot exceed the product of blood flow rate and the chemical concentration in arterial blood. The actual rate of chemical accumulation at any point in time depends on both the prior exposure history (i.e., the extent to which chemical has already accumulated) and the rate of chemical exchange between blood and tissues, both of which have the potential to impact C_{vi}.

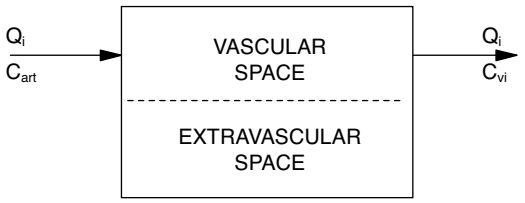

FIGURE 3.23 Schematic representation of a compartment exhibiting flow-limited kinetics. Symbols: Q_i, tissue blood flow rate; C_{art}, chemical concentration in arterial blood; C_{vi}, chemical concentration in venous blood draining the tissue.

A relatively simple tissue description can be developed if it is assumed that the rate of chemical exchange between blood and tissue is fast relative to the blood perfusion rate (Figure 3.23). Under these circumstances, chemical partitioning proceeds to equilibrium with the result that C_{vi} is determined by the chemical concentration in the tissue and the relative affinity of the compound for the tissue and for blood, denoted as P_i, the equilibrium tissue–blood partition coefficient:

$$C_{vi} = C_i / P_i \tag{3.84}$$

Substituting Equation 3.84 into Equation 3.83, one obtains:

$$dX_i/dt = Q_i\left(C_{art} - C_i/P_I\right) \tag{3.85}$$

or equivalently:

$$V_i dC_i/dt = Q_i\left(C_{art} - C_i/P_i\right) \tag{3.86}$$

where C_i is the chemical concentration in the tissue, and V_i is the tissue volume. This type of chemical distribution is said to be flow limited because the rate of chemical accumulation is controlled by the blood flow rate.

Chemical distribution is said to be diffusion limited when the rate of chemical diffusion across a biological membrane limits chemical flux between blood and tissues. Under these circumstances, the tissue must be divided into two subcompartments (Figure 3.24). Chemical flux between subcompartments is then modeled using the Fick relationship (see Equation 3.1). In practice, chemical permeability (i.e., DP_{mw}/h) and the surface area for diffusion are difficult to determine. The permeability-area (PA) product is therefore frequently treated as a first-order transport parameter (k_i), the value of which is determined from exposure data. If a diffusion limitation exists at the capillary endothelium, the relevant subcompartments are the vascular and extravascular tissue spaces. Alternatively, if the diffusion limitation exists at the cellular membrane, the relevant subcompartments are the extracellular and intracellular spaces. In either case, the mathematical treatment is identical, but different volumes must be assigned to represent each subcompartment.

For illustration, it is assumed here that the diffusion limitation exists at the capillary endothelium. Each subcompartment is considered to be a well-mixed phase; therefore, venous blood exiting the tissue has the same chemical concentration as that of the vascular subcompartment space. With these assumptions, a mass-balance equation for the extravascular space (ei) may written as:

$$V_{ei} dC_i/dt = k_i\left(C_{vi} - C_{ei}\right) \tag{3.87}$$

and for the vascular space (bi) may written as:

$$V_{bi} dC_{bi}/dt = Q_i\left(C_{art} - C_{vi}\right) - k_i\left(C_{vi} - C_{ei}\right) \tag{3.88}$$

The chemical concentration in the whole tissue may then be calculated from the volume-weighted contributions of the vascular and extravascular spaces:

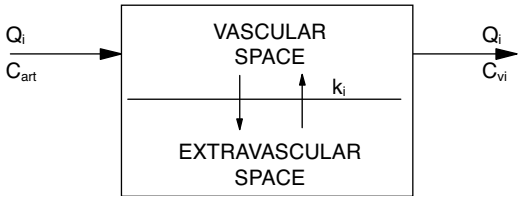

FIGURE 3.24 Schematic representation of a compartment exhibiting diffusion-limited kinetics. Symbols: Q_i, tissue blood flow rate; C_{art}, chemical concentration in arterial blood; C_{vi}, chemical concentration in venous blood draining the tissue; k_i, transport parameter used to model diffusive flux across the capillary endothelium.

$$C_i = \left(V_{bi}C_{bi} + V_{ei}C_{ei}\right)/V_i \tag{3.89}$$

In these equations, the terms C_a, C_{vi}, C_{bi}, C_{ei}, and C_i refer to *diffusible* chemical concentrations. Operationally, this includes both freely dissolved chemical and chemical that is loosely bound, such that the kinetics of desorption do not limit exchange. In some cases, it may be possible to estimate the concentration of diffusible chemical by using a static technique such as ultrafiltration to measure bound and free compound. If, however, desorption of bound chemical contributes to reestablishing the gradient for chemical flux, these data will underestimate the true concentration of diffusible chemical. One way to overcome this problem is to assume that the percentage of total chemical in both the vascular and extravascular spaces that is in a diffusible form is the same. The magnitude and direction of the concentration gradient for diffusion can then be calculated as the difference between chemical concentration in the vascular space and that in the extravascular space, divided by a tissue–blood equilibrium partition coefficient. Under these conditions, Equations 3.86 and 3.87 may be rewritten as:

$$V_{ei}dC_i/dt = k_i\left(C_{vi} - C_{ei}/P_i\right) \tag{3.90}$$

and

$$V_{bi}dC_{bi}/dt = Q_i\left(C_{art} - C_{vi}\right) - k_i\left(C_{vi} - C_{ei}/P_i\right) \tag{3.91}$$

where all concentration terms refer to total chemical concentrations.

Strong chemical binding interactions add complexity to these relatively simple formulations; for example, a number of specific binding proteins sequester metals in tissues, limiting their toxicity (Roesijadi and Robinson, 1994). Similarly, some organic compounds are highly bound by serum albumin while others form stable complexes with specific intracellular receptors. Low-capacity binding systems often follow a Langmuir-type isotherm, saturating at high substrate concentrations. Under these circumstances, the total concentration of chemical in the relevant compartment (C_j; here, denoting C_i, C_{ei}, or C_{bi}) can be expressed as the sum of free (C_j^*) and bound compound (expression in parentheses):

$$C_j = C_j^* + \left(\alpha_i C_j^* / \left(\varepsilon_j + C_j^*\right)\right) \tag{3.92}$$

where α_j is the binding capacity of the receptor and ε_j is the receptor dissociation constant. A first-order binding coefficient may be sufficient to describe the kinetics of a high-capacity binding system.

In view of these potential complexities, it is perhaps remarkable that the assumption of flow-limited chemical distribution has been shown to approximate the kinetic behavior of a large number of compounds in both fish and mammals. The principal advantage of this assumption is that it does not require the fitting of any kinetic parameters. For this reason, it is often used as a default assumption in model formulation. Exposure data are then examined for evidence of a diffusion limitation on chemical flux, generally presented as a slower than anticipated rate of accumulation. Such data would be insufficient to determine where the diffusion limitation existed and to what extent, if any, strong binding played a role in retarding uptake. It could be concluded, however, that additional studies are needed to guide model development. Factors that contribute to diffusion-limited chemical distribution include those generally associated with

slow diffusion across biological membranes: large molecular volume, the existence of charged or highly polar substituent groups, and extreme hydrophobic character. The specific rate (per gram of tissue) of tissue blood perfusion may also be a factor. Low blood perfusion rates are associated with large intercapillary diffusion distances. Moreover, in a tissue with very low metabolic demand (e.g., the white muscle of a resting fish), only a fraction of the total tissue mass may be perfused at any point in time. In mammals, diffusion limitations have also been observed in highly perfused tissues, presumably because of reduced blood residence times. In modeling efforts with fish conducted to date, diffusion limitations have been employed to describe the uptake of pyrene into muscle tissue of rainbow trout (Law et al., 1991) and the accumulation of TCDD in adipose tissue of brook trout (Nichols et al., 1998). In the brook trout model, the PA product was calculated as a fraction of the estimated fat blood flow rate. The fitted value of the PA was then interpreted as the effective rate of blood flow to fat.

As the kidney and liver of fish receive both arterial and portal blood, a simple description of these organs can be developed by assuming that arterial and portal blood mix before exchanging with the tissue. Under these circumstances, total blood flow (Q_{mi}) equals the sum of arterial (Q_i) and portal inputs (Q_{pi}):

$$Q_{mi} = Q_i + Q_{pi} \tag{3.93}$$

where Q_{pi} is equal to all or a fraction of arterial flow to the upstream compartment. The chemical concentration in mixed blood (C_{mi}) can be calculated by summing the flow-weighted contributions of arterial and portal blood (C_{pi}):

$$C_{mi} = \left(Q_i C_{art} + Q_{pi} C_{pi}\right) / Q_{mi} \tag{3.94}$$

where C_{pi} is equal to the concentration in venous blood exiting the upstream compartment. Assuming further that chemical uptake by the tissue is flow limited, a mass balance for the compartment may then be written as:

$$dX_i/dt = Q_{mi}\left(C_{mi} - C_{vi}\right) \tag{3.95}$$

When the kinetic descriptions for all tissues have been defined, the concentration of chemical in mixed venous blood can be calculated from the flow-weighted contributions of venous blood draining each tissue compartment. In a model with chemical uptake at the gills, this mixed venous concentration provides one of the inputs to the branchial exchange description (see below). The complete model consists of a system of simultaneous mass-balance differential equations. Numerical integration procedures are then used to solve these equations at each time point.

Routes of Exposure

A critical part of any PBTK model is the route of exposure description. In experimental dosing studies, the investigator generally controls the route and timing of the exposure. Natural exposures may be more difficult to characterize and are more likely to be of a mixed type—that is, involving chemical flux at more than one exchange surface; for example, a compound that is present in water may also be present in prey items upon which a fish feeds. Environmental routes of uptake (branchial, dermal, dietary) can also function as routes of elimination. Indeed, it is possible for one exchange surface to function as a route of uptake (e.g., the gastrointestinal tract), while at the same time another operates as a route of elimination (e.g., the gills). In contrast, some experimental routes of exposure, such as intravascular infusion, function only as routes of uptake.

Branchial Uptake

The structure of fish gills reflects their primary function as a gas-exchange and osmoregulatory organ. A large surface area for exchange is achieved by an elaboration of plate-like structures termed *lamellae*, which form narrow channels above and below each gill filament. In most species, blood flows through

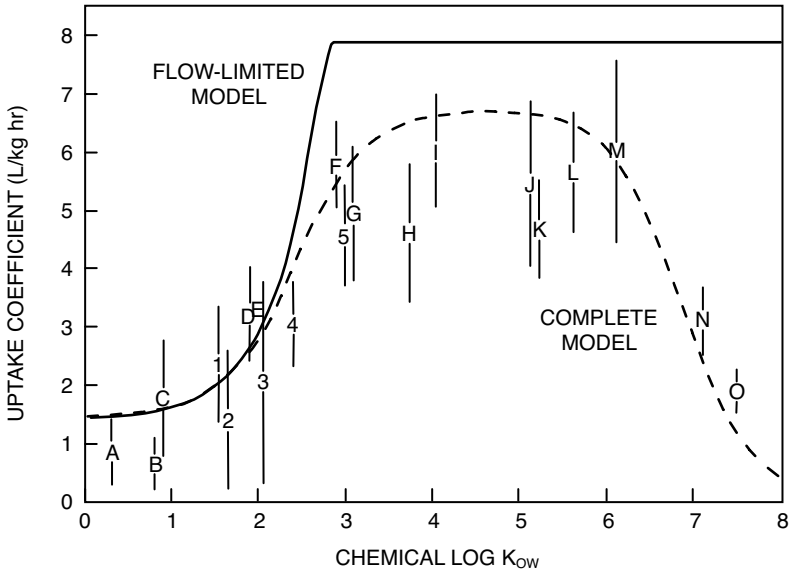

FIGURE 3.25 Observed and predicted uptake of chemicals across the gills of adult rainbow trout as a function of chemical log K_{ow}. Branchial uptake predicted by the flow-limited gill model is shown as a solid line; that predicted by the gill model with flow and diffusion limitations is shown as a dashed line. (Adapted from Erickson, R.J. and McKim, J.M., *Aquat. Toxicol.*, 18, 175–198, 1990.)

the lamellae in a posterior-to-anterior direction, providing for highly efficient counter-current exchange with inspired water.

The principal limitations on chemical uptake at fish gills were reviewed earlier in this chapter (see Compartmental Models for Fish and Branchial Clearance sections). If the permeability of the gill epithelium to a compound is low, diffusion can limit exchange, and uptake flux is controlled by the product of gill permeability and the surface area for diffusion. If, on the other hand, the permeability of the epithelium is high, uptake may be limited by the chemical capacities of blood and water flowing to the gills. A blood flow limitation exists if more chemical is delivered to the gills in respiratory water than can be carried away in blood. A water flow limitation exists if the capacity of blood to carry chemical away from the gills exceeds the chemical capacity of respiratory water.

Ignoring diffusion limitations on uptake, Erickson and McKim (1990a) developed a model for the branchial flux (F_g) of organic chemicals based on flow limitations and the behavior of counter-current exchange systems. The complete flow-limited model may be stated as:

$$F_g = \text{Min}\left[Q_w, Q_c P_{bw}\right]\left[C_w - \left(C_v / P_{bw}\right)\right] \tag{3.96}$$

where Q_w is the flow of inspired water that exchanges with blood, Q_c is the total cardiac output, P_{bw} is an equilibrium blood–water partition coefficient, and C_v is the chemical concentration in venous blood entering the gills. In this equation, F_g is calculated as the product of the concentration gradient between inspired water and venous blood entering the gills (second term in brackets) and the chemical capacity of blood or respiratory water (first term), whichever is less. When the flow-limited model was parameterized using data for rainbow trout it reproduced the well-known dependence of branchial uptake efficiency on chemical log K_{ow} for compounds of low to intermediate hydrophobicity but did not predict the observed decline in uptake for high log K_{ow} compounds (Figure 3.25). In addition, the model tended to overestimate the maximal rate of uptake by about 20%.

Subsequently, Erickson and McKim (1990b) published a more comprehensive gill model that included both flow and diffusion limitations, as well as binding of high log K_{ow} compounds to dissolved organic material. The full model, which included advective (direction of fluid flow) and diffusive (perpendicular

to fluid flow) chemical transport in both blood and water, did not yield an analytical solution. An approximate analytical solution was obtained, however, by segregating the lamellar unit into layers in which only advective or diffusive transport predominates. This may be visualized as a diffusion barrier consisting of the gill epithelium and stagnant boundary layers in the adjacent water and blood channels. With these simplifications, branchial flux can be calculated as the product of the concentration gradient between inspired water and venous blood and an exchange coefficient (k_x):

$$F_g = k_x \left(C_w - C_v P_{bw}\right) \tag{3.97}$$

where

$$k_x = \frac{e^{-kd/kb} - e^{-kd/kw}}{\frac{e^{-kd/kb}}{k_w} - \frac{e^{-kd/kw}}{k_b}} \tag{3.98}$$

The terms k_w, k_b, and k_d in the equation for k_x represent the capacity of respiratory water, blood flowing through the gills, and chemical diffusion across the gills, respectively, to support branchial flux:

$$k_w = Q_w \tag{3.99}$$

$$k_b = Q_c P_{bw} \tag{3.100}$$

$$k_d = DA/h \tag{3.101}$$

where D, A, and h, respectively, refer to the chemical diffusivity in, the total area of, and the effective thickness of the gill diffusion barrier. Importantly, this model assumes that chemical diffusion in the aqueous phase of the gill epithelium limits flux. The model does not, therefore, incorporate the partition coefficient (P_{mw}), which appears in the model given by Hayton and Barron (1990) (Equation 3.27). A simulation for rainbow trout generated using the complete model (approximate analytical solution) is shown in Figure 3.25. This simulation accurately describes all of the observed trends in the data, including the reduction in uptake of high log K_{ow} compounds.

The complete gill model has been used to describe branchial uptake of waterborne chemicals by several fish species, requiring the collection of necessary physiological and gill morphometric information (Figure 3.26) (Lien et al., 2001; Nichols et al., 1993). For many compounds, however, a good approximation of branchial flux can be obtained using the simpler flow-limited model. Direct experimental evidence for the predominant influence of flow limitations on branchial uptake has been obtained by independently varying Q_w and Q_c for chemicals with different log K_{ow} values (Schmieder and Weber, 1992). Finally, it is important to understand that both the flow-limited and complete gill models separate the blood circulation into arterial and venous sides. In a PBTK model, chemical taken up across the gills (predicted by the gill description) is added to that already present in venous blood to provide a new value for the arterial blood concentration. The arterial blood concentration is then used as an input to each of the tissue descriptions (Figure 3.22).

Dermal Uptake

Dermal uptake represents a second possible route of exposure that may be important for small fish, juveniles of larger species, and fish that live in intimate contact with contaminated sediments. The structure and function of fish skin were addressed earlier in this chapter. Additional information is provided in a recent review (McKim and Lien, 2001). The general architecture of fish skin (epidermis, dermis, and hypodermis) is similar in most species, but the thickness of these layers varies widely. Additional differences exist with respect to the presence or absence of scales and the number and location of sensory organs, mucous glands, and other specialized structures.

A PBTK model for dermal uptake in small fish was developed by Lien and co-workers (Lien and McKim, 1993; Lien et al., 1994) from the gill model given in Equation 3.97 by assuming that water flow does not limit chemical transport across the skin. This model can be visualized as a parallel network

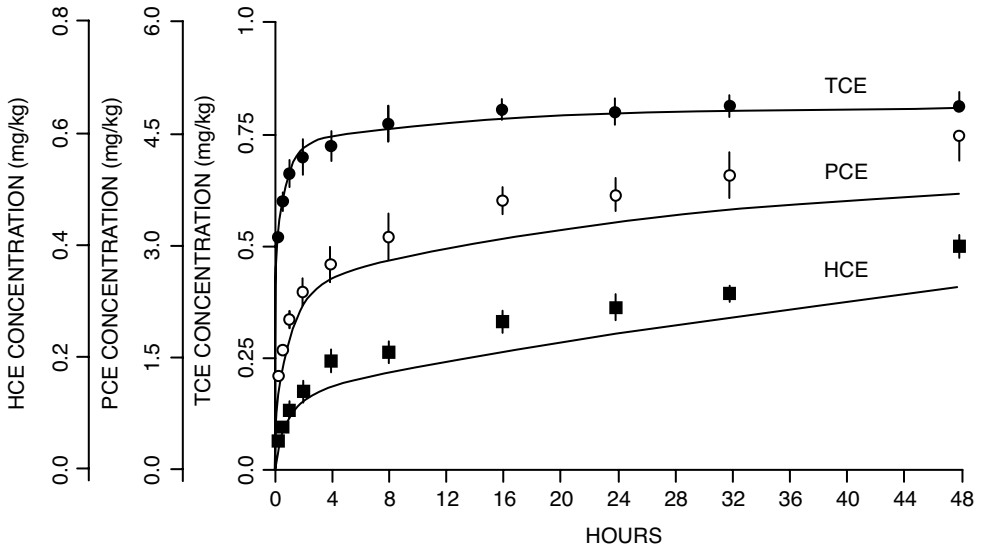

FIGURE 3.26 Concentration time course of tetrachloroethane (TCE), pentachloroethane (PCE), and hexachloroethane (HCE) in arterial blood of adult channel catfish. Catfish were simultaneously exposed to all three chloroethanes in water. Solid lines represent simulations generated using a PBTK model incorporating the branchial uptake description given by Erickson and McKim (1990b). Measured values are shown as individual points. (Adapted from Nichols, J.W. et al., *Aquat. Toxicol.*, 27, 83–112, 1993.)

of capillaries with chemical diffusion from water to blood perpendicular to the capillary axis. Given these assumptions Equation 3.97 simplifies to:

$$k_x = k_b\left(1 - e^{-kd/kb}\right) \tag{3.102}$$

This description suggests that dermal flux may be limited by the capacity of the skin surfaces to support chemical diffusion (k_d) and the capacity of blood flowing to the skin (k_b) to remove chemical once it has been absorbed. The terms k_b and k_d are analogous to those given in Equations 3.99 and 3.100, except that in this case diffusivity, area, and thickness refer to the skin diffusion barrier, and the relevant blood flow rate is that to skin. As in the gill model given by Erickson and McKim (1990b), diffusive flux was assumed to be limited by chemical diffusion in the aqueous phase of the diffusion barrier. Using this model, dermal flux (F_s) may be calculated as the product of k_x and the concentration gradient for uptake:

$$F_s = k_x\left(C_w - C_a/P_{bw}\right) \tag{3.103}$$

The chemical concentration in venous blood draining the skin is then calculated by adding this flux to the amount of chemical already present in arterial blood perfusing the skin.

This model was used to compare the capacities of gill and skin surfaces to support chemical uptake in small fish. Lien and McKim (1993) exposed fathead minnows and Japanese medaka to 2,2′,5,5′-tetrachlorobiphenyl (TCB), and Lien et al. (1994) exposed fathead minnows to a homologous series of three chlorinated ethanes. Model predictions (gill only and gill plus skin) were evaluated by comparison with measured whole-body chemical residues. Based on this analysis, approximately 50% of the total uptake of TCB was attributed to dermal absorption. The contribution of dermal absorption to the uptake of the three chloroethanes ranged from 20 to 30% and tended to increase with the degree of chlorine substitution.

A more complex description of dermal uptake is required if chemical accumulation by skin tissue impacts chemical flux between water and blood. Following the approach used by McDougal et al. (1990) to describe dermal flux of chemical vapors by rats, Nichols et al. (1996) developed a dermal uptake model for fish that incorporates a discrete skin compartment. The model structure was also changed somewhat

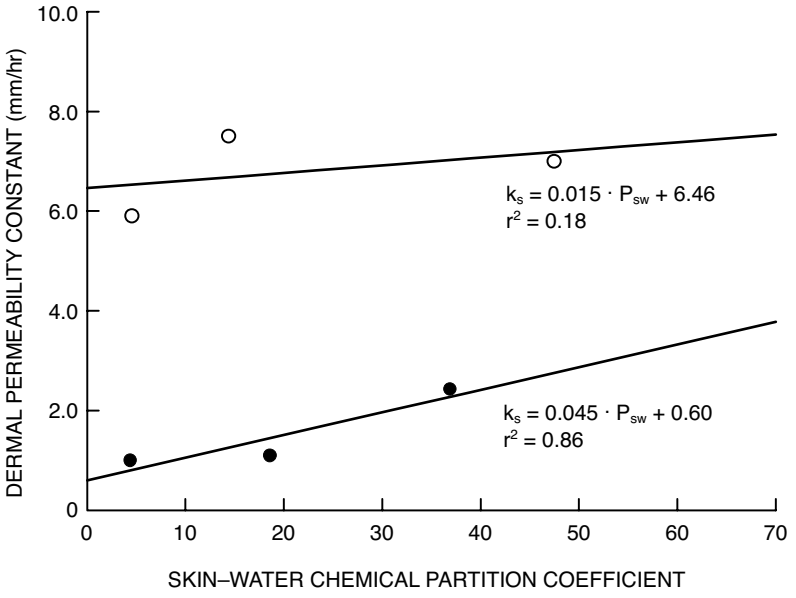

FIGURE 3.27 Dermal permeability of three chlorinated ethanes in rainbow trout and channel catfish as a function of skin–water chemical partitioning. Fitted permeability coefficients for rainbow trout are given as solid circles. Open circles represent fitted coefficients for channel catfish. Equations were generated for each species by simple linear regression. (Adapted from Nichols, J.W. et al., *Fundam. Appl. Toxicol.*, 31, 229–242, 1996.)

from that of Lien et al. (1994) to reflect more detailed aspects of vascular organization. Specifically, anatomical studies with fish suggest that, although a portion of venous blood draining the skin contributes directly to the mixed venous return, most of this blood flows to the caudal vein and from there to the kidney.

Assuming that chemical transfer between blood and skin is flow limited, a mass balance on the skin compartment may be written as:

$$V_s dC_s/dt = Q_s\left(C_a - C_s/P_s\right) + k_s A_s\left(C_w - C_s/P_{sw}\right) \tag{3.104}$$

where P_s and P_{sw} are skin–blood and skin–water equilibrium partition coefficients. The first term on the right-hand side of this equation is identical to that given previously for a flow-limited tissue (Equation 3.86), and the second term describes chemical diffusion across a membrane separating skin tissue from the external environment. In this formulation, k_s is the permeability coefficient defined as in Equation 3.1 (DP_{mw}/h), and A_s is the total skin surface area.

The dermal submodel was evaluated by exposing large trout and channel catfish to a series of chlorinated ethanes in a chambered exposure system (McKim et al., 1996). For these studies, only the trunk region of the fish was exposed. Chemical uptake across the skin then resulted in the accumulation of chemical in blood and tissues and elimination across the gills. By using compounds for which validated inhalation models already existed, it was possible to fit a set of apparent skin permeability coefficients (k_s) (Nichols et al., 1996). Figure 3.27 shows fitted permeability constants for three chloroethanes in both trout and channel catfish. Fitted permeability coefficients increased little if at all with increasing chemical affinity for skin. Based on this observation, the authors speculated that diffusion through tissue water limits dermal flux of moderately hydrophobic compounds.

Dietary Uptake

Chemical and physiological factors that control xenobiotic uptake from the diet were described earlier in this chapter. The dietary route of exposure may be important within an aquaculture setting as a means of dosing animals with antibiotics and other therapeutic agents. Oral bioavailability defined using AUC

methods is often used to characterize uptake efficiency for these compounds. The diet also represents an important route of uptake for lipophilic environmental contaminants (log $K_{ow} > 5$) (Bruggeman et al., 1984). This is due in part to the fact that these compounds accumulate to high levels in prey items. High log K_{ow} compounds also tend to bind to dissolved and particulate organic material in water, reducing their availability for branchial uptake. Similar considerations may also apply to several metalloids and organometallic compounds, including selenium, cesium, methylmercury, and tributyltin.

Considerable effort has been expended to identify factors that control dietary uptake of hydrophobic compounds, and recent modeling work reflects this emphasis. Within the intestine, uptake of dietary lipid and a reduction of meal volume increase chemical activity in the gut contents above that of the meal, potentially resulting in chemical uptake by fish even when chemical concentrations in their prey are at or near equilibrium with those in water (see Mechanism of Biomagnification section below). It is important, however, to note that even low rates of metabolism could reduce the extent of chemical accumulation substantially (Clark et al., 1990; de Wolf et al., 1992; Endicott and Cook, 1994; Nichols et al., 2004b). Chemicals in the diet are particularly susceptible to metabolic clearance because of the potential for presystemic metabolism in tissues of both the gut and liver (Van Veld, 1990).

To date, very few PBTK models incorporating a dietary uptake description have been developed for mammals, and only two have been published for fish. Most of the mammalian models have treated dietary uptake as a first-order absorptive process. If absorption is assumed to be 100% efficient, the dose can be modeled as a mass of compound and it is not necessary to define a gut compartment. A second approach also employs a first-order absorptive rate constant but provides for the possibility that absorption is less than 100% efficient. In this second approach, a gut lumen compartment is defined and the consumption of food and egestion of feces are modeled as bulk flow rates or periodic events. In both of these approaches, the value of the first-order rate constant is generally obtained by fitting model simulations to measured (usually blood or plasma) chemical concentrations.

At the other end of the spectrum in terms of complexity, Bungay et al. (1981) developed a highly detailed model for dietary uptake of chlordecone in the rat. The gut portion of this model consists of six compartments corresponding to the stomach, small intestine (three compartments), cecum, and lower intestine. Diffusion limitations on chemical flux were assumed to exist at both the tissue–blood and tissue–gut lumen interfaces, and 12 different rate constants were fitted by modeling to measured residues in tissues and gut contents.

Nichols et al. (1998) used two approaches to model the accumulation of TCDD in feeding studies with brook trout. The first approach was based on the assumption that a chemical equilibrium is established between fecal material contained within the terminal colon (C_{fec}) and venous blood draining the GIT (C_{vgt}):

$$C_{fec} = C_{vgt}P_{fb} \tag{3.105}$$

where P_{fb} is the feces–blood equilibrium chemical partition coefficient. Termed the *fecal partitioning submodel*, this approach is similar to that proposed by Barber et al. (1991) to model PCB accumulation by lake trout, the principal difference being that an equilibrium is established with venous blood exiting the gut and not with the entire fish.

From mass-balance considerations, it follows that:

$$\left(Q_{food}C_{food}\right) - \left(Q_{fec}C_{fec}\right) = \left(Q_{gt}C_{vgt}\right) - \left(Q_{gt}C_{art}\right) \tag{3.106}$$

where Q_{food} is the feeding rate, C_{food} is the TCDD concentration in the diet, Q_{fec} is the fecal egestion rate, Q_{gt} is the blood flow rate to the lower intestines, and C_{art} is the chemical concentration in arterial blood. Substituting Equation 3.105 into Equation 3.106 and solving for C_{vgt} yields:

$$C_{vgt} = \left(Q_{gt}C_{art} + Q_{food}C_{food}\right) / \left(Q_{gt} + Q_{fec}P_{fb}\right) \tag{3.107}$$

Venous blood draining the tissues of the lower intestine was then assumed to mix with arterial blood flowing to the liver.

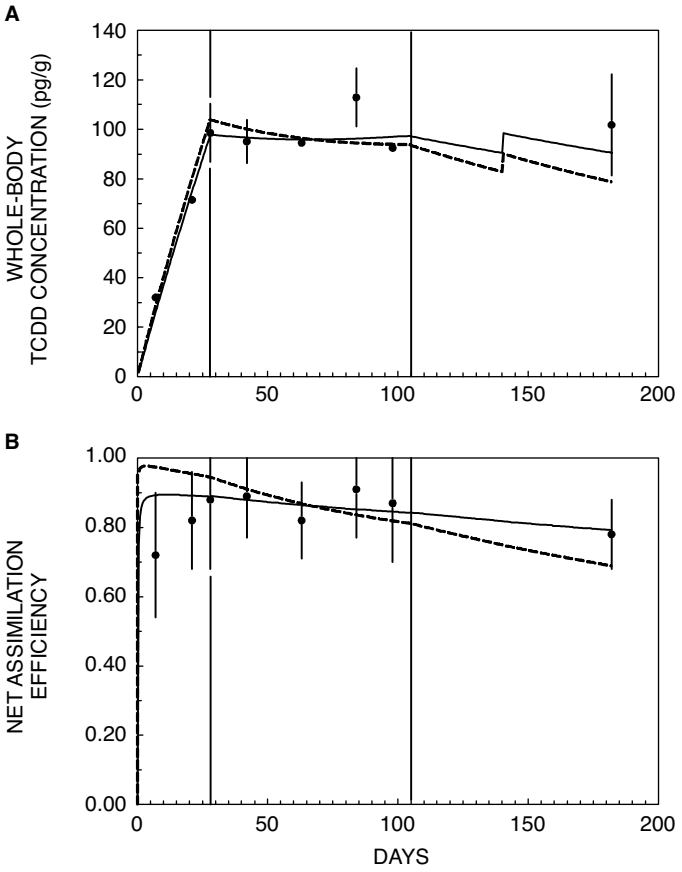

FIGURE 3.28 Accumulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in brook trout. Measured whole-body concentrations (A) and calculated assimilation efficiencies (B) are shown as individual points. Simulations were obtained using a physiologically based toxicokinetic model for brook trout. Dashed lines were generated using a "fecal partitioning" gut submodel. Solid lines were obtained by imposing a "diffusion limitation" on dietary uptake. Vertical lines delineate the three phases of the study: loading (0 to 28 days), maintenance (29 to 105 days), and depuration (105 to 182 days). (Adapted from Nichols, J.W. et al., *Environ. Toxicol. Chem.*, 17, 2422–2434, 1998.)

The primary advantage of this simple gut description is that it is not necessary to fit the value of a first-order absorption rate constant. In a very simplistic way, the model also accounts for changes in chemical affinity that accompany digestion (through its impact on P_{fb}) and the concentrating effect of a reduction in meal volume (by specifying both Q_{food} and Q_{fec}). Moreover, because chemical is allowed to accumulate in blood flowing to the gut tissue compartment, the model provides for the possibility of decreased absorption efficiency due to a decline in the gradient for uptake. The disadvantage of this model is that it cannot account for kinetic limitations that may prevent an equilibrium from being established between feces and blood.

In the brook trout study, measured whole-body TCDD residues were lower than those predicted by the fecal partitioning model, suggesting the existence of a kinetic limitation on dietary uptake (Figure 3.28). A second model was therefore developed that incorporated a diffusion limitation on the chemical flux between feces and tissues of the intestinal tract. For the feces:

$$dA_{fec}/dt = \left(Q_{food}C_{food}\right) - \left(Q_{fec}C_{fec}\right) - k_{gt}\left(C_{fec} - C_{gt}P_{fgt}\right) \tag{3.108}$$

where A_{fec} is the amount of TCDD in feces, k_{gt} is a first-order rate constant, C_{gt} is the chemical concentration in gut tissues, and P_{fgt} is the feces–gut tissue equilibrium partition coefficient. For tissues of the intestinal tract:

$$dA_{gt}/dt = Q_{gt}\left(C_{art} - Cv_{gt}\right) + k_{gt}\left(C_{fec} - C_{gt}P_{fgt}\right) \quad (3.109)$$

Chemical concentration in venous blood draining the gut was then determined from that of the gut tissues by assuming that chemical exchange between blood and tissues was flow limited.

The value of k_{gt} was determined by Nichols et al. (1998) by fitting model simulations to measured whole-body TCDD concentrations and calculated assimilation efficiencies. The model was then used to simulate TCDD kinetics in various tissues and organs, resulting in good correspondence between predicted and observed values. The disadvantage of this approach is that k_{gt} is difficult to interpret. The fitted value of k_{gt} (which has units of flow) was found to be about 5% of the estimated blood flow rate to the lower intestine. It could not be determined, however, whether this reduction in uptake was due to a limitation on diffusion, chemical binding in feces, or some other kinetic constraint.

Recently, Nichols et al. (2004a) developed a more detailed description of the fish GIT to describe dietary uptake of [^{14}C]-2,2′,5,5′-tetrachlorobiphenyl (TCB) by rainbow trout. The GIT was modeled using four compartments corresponding to the stomach, pyloric ceca, upper intestine, and lower intestine, and the luminal volume of each compartment was allowed to change in time as a function of bulk flow of ingesta and nutrient uptake. The model was developed using data from rainbow trout that were fed a meal of 60-day-old fathead minnows contaminated with TCB. Chemical partitioning coefficients were adjusted to account for changes in chemical affinity associated with the uptake of dietary lipid. Permeability coefficients for the absorbing gut segments were then fitted by modeling to measured TCB concentrations in gut contents and tissues.

As expected, most of the TCB was taken up within the upper intestine during the period of peak lipid absorption. It was concluded, however, that a kinetic limitation on chemical uptake acting along the entire length of the GIT resulted in a chemical disequilibrium between feces and tissues of the lower intestine. The mechanistic basis for this kinetic limitation remains unknown; however, a comparison of fitted gut permeability coefficients with the permeability coefficient for branchial uptake of TCB suggests that dietary uptake is unlikely to be limited by the rate of diffusion across the gastrointestinal epithelium.

The gut model was then used to simulate chronic exposures to TCB and a series of hypothetical high log K_{ow} compounds (Nichols et al., 2004b). Predicted steady-state biomagnification factors for TCB were very close to values measured in both laboratory and field studies; however, the incorporation of a log K_{ow}-dependent decrease in gut permeability was required to reproduce observed trends in biomagnification and dietary assimilation efficiency. Although not constituting proof as such, this finding suggests that dietary absorption of hydrophobic organic compounds by fish is controlled in part by factors that vary with chemical log K_{ow}.

Hepatic and Renal Elimination

The foregoing descriptions of chemical exposure routes in fish are bidirectional with respect to chemical flux and can function, therefore, as routes of elimination when activity gradients favor the movement of chemical out of the organism. Thus, blood and water flow limitations that define maximum rates of chemical uptake at the gills will also act to limit maximal rates of branchial elimination. Depending, however, on the way that a particular compound is handled, a complete PBTK model may also have to incorporate other routes of elimination, potentially including urinary and biliary elimination, as well as biotransformation.

The physiological and biochemical factors that dictate hepatic and renal handling of xenobiotic compounds were reviewed earlier in this chapter. Although limited, studies in fish suggest that hepatic and renal clearance contribute substantially to the elimination of many compounds, particularly if they are substrates for active systems that secrete chemicals to urine and bile. Currently, guidance on the kinetics of elimination by urinary and biliary routes comes largely from mammalian studies. For most compounds, the kinetics of renal clearance by glomerular filtration are first order with respect to the free chemical concentration in plasma. In contrast, clearance pathways involving active secretion to urine or bile often exhibit saturable, nonlinear kinetics. Biotransformation pathways in both mammals and fish may also exhibit nonlinear kinetics.

The first step in modeling elimination is to specify the organs where it occurs and the chemical kinetics (e.g., first-order, saturable) over the concentration range to which the tissue is exposed. Elimination may then be modeled by incorporating a clearance expression (dA_{cl}/dt) into the mass-balance equation for the eliminating compartment:

$$dA_i/dt = Q_i\left(C_{art} - C_{vi}\right) - dA_{cl}/dt \tag{3.110}$$

Referenced to the chemical concentration in venous blood draining the tissue, the kinetics of first-order clearance can be written as:

$$dA_{cl}/dt = k_{fc}\,C_{vi}V_i \tag{3.111}$$

Saturable pathways can be described using a Michaelis–Menten type of equation where K_m and V_{max} are parameters that define an *in vivo* intrinsic clearance term:

$$CL_{int} = V_{max}/\left(K_m + C_{vi}\right) \tag{3.112}$$

$$dA_{cl}/dt = V_{max}C_{vi}/\left(K_m + C_{vi}\right) = CL_{int}C_{vi} \tag{3.113}$$

Although often localized to the liver as a matter of convenience, biotransformation can be incorporated into any one or more of the individual tissue descriptions. Ascribing metabolism to the tissue where it actually occurs may be critical to the modeling outcome when this activity limits chemical uptake across the gills or gut (Barron et al., 1989; Van Veld, 1990). In some cases, a modeler may be more interested in the kinetics of a metabolite than of the parent compound from which it was derived. Under these circumstances, the disappearance of parent compound (including both the rate of metabolism and the site where this metabolism occurs) is equated to the production of the metabolites) (Nichols, 1999). Additional information, including equilibrium partitioning, plasma binding, chemical reactivity (e.g., covalent binding to tissue macromolecules), and subsequent elimination, is then required to develop a description of metabolite kinetics.

Model Parameterization

Anatomical, physiological, and biochemical information required to develop PBTK models for fish is given throughout this text. Additional information is provided in numerous texts and hundreds of scientific papers. Although it is true that most of this information has been developed for a relatively few fish species, allometric and temperature relationships permit the extrapolation of these data to other, untested species. The goal of this section is to provide guidance on how existing data can be used in PBTK modeling efforts. An emphasis is placed on chemical elimination pathways, as these pathways are often found to be primarily responsible for differences in chemical disposition that exist among species.

Physiological Inputs

In the absence of measured values for a given fish species, it may be possible to estimate the values of critical physiological inputs using allometric scaling relationships. A review of allometric scaling techniques is given by Schmidt-Nielsen (1984). These principals have been applied to PBTK modeling efforts by several authors (Dedrick, 1973; Mordenti, 1986; Rowland, 1985; Travis et al., 1990). In mammals, physiological parameters linked to cellular metabolism (e.g., cardiac output, oxygen consumption rate) often scale to the 3/4 power of body weight (exponent of 0.75). Renal clearance and the capacity for hepatic metabolism (V_{max}) were reported by Travis et al. (1990) to scale to an exponent of 0.75. In a more recent review, Hu and Hayton (2001) found that renal clearance scales to the 2/3 power of body weight (exponent of 0.67). The Michaelis–Menten constant, K_m, which is a measure of the substrate affinity of an enzyme, is thought to remain relatively constant across species.

In studies with fish, it has been difficult to make comparisons among species because of differences in experimental design (e.g., temperature, light cycle) that affect metabolic rate. The strongest comparisons,

therefore, have been made using different sized individuals of a single species held under otherwise identical conditions. Although highly variable among species, the average allometric exponent for oxygen consumption rate is about 0.8, or slightly higher than the average value for mammals (Schmidt-Nielsen, 1984). Within a single species, gill surface area also tends to scale to an exponent of about 0.8 (Hughes, 1984), as does the absorptive surface area of the intestinal mucosa (Buddington and Diamond, 1987). Skin surface area within a species scales to an exponent of about 0.67 in accordance with the surface law (Schmidt-Nielsen, 1984). Data presented by Wood and Shelton (1980) suggest that cardiac output in rainbow trout scales to an exponent of about 0.9. The dependence of cardiac output on acclimation temperature also has been characterized for rainbow trout (Barron et al., 1987a). Cardiac output in trout had a Q10 of 4.0 (i.e., a 10°C increase in temperature was associated with a fourfold increase in cardiac output). Q10 values reported for oxygen consumption in fish and other poikilotherms generally average around 2.0 (Ott et al., 1980).

Small fish present special challenges for PBTK modeling because direct measurements of many anatomical and physiological parameters are difficult or impossible to make. Lien and McKim (1993) addressed this problem in studies with Japanese medaka and fathead minnows by calculating effective respiratory volume (Q_w) and functional gill surface area as functions of oxygen consumption rate, which can be easily measured even in larval fishes. Cardiac output for these small fish species was estimated using a relationship given by Erickson and McKim (1990b) that accounts for the effect of both size and temperature.

Chemical Partitioning

Mass-balance equations that describe chemical flux between blood and tissues include a term that defines both the direction and magnitude of the concentration gradient. Generally, the blood is defined as the reference state. The concentration gradient is then calculated as the difference between the chemical concentration in blood and the concentration in the tissue, divided by an equilibrium tissue–blood partition coefficient. In the context of this description, it is assumed that chemical associations with blood and tissue constituents are low affinity. Operationally, this means that the kinetics of chemical dissociation from binding sites in blood and tissues are fast relative to the residence time of blood in tissues and do not, therefore, limit the overall kinetics of distribution. Partition coefficients are also required to develop physiological descriptions of chemical flux at the gills, skin, and gut.

Chemical partition coefficients can be obtained directly from chemical concentrations in blood and tissues of animals that have been exposed to near steady state, provided that metabolism or some other route of elimination does not reduce tissue concentrations below those expected from simple partitioning. The disadvantage of this approach is that it requires prior exposure of animals to generate model simulations. A number of *in vitro* systems have also been developed to generate partition coefficients. Gargas et al. (1989) used a vial headspace equilibration technique to determine partition coefficients for a large number of volatile hydrocarbons. This method was subsequently adapted for use with fish tissues (Hoffman et al., 1992). The vial equilibration method measures the depression in headspace concentration that occurs as a result of chemical partitioning to the sample. Samples generally consist of tissue homogenates diluted in saline, although the technique has also been adapted for use with intact samples of skin (Mattie et al., 1993). Other *in vitro* partitioning methods have been developed for nonvolatile compounds. Law et al. (1991) obtained partitioning estimates for pyrene in trout tissues using an equilibrium dialysis technique. Jepson et al. (1994) used a filtration method to evaluate the partitioning of lindane, parathion, paraoxon, perchloroethylene, and two haloacetic acids between rat tissues and saline. Murphy et al. (1995) measured the partitioning of TCDD and estradiol between rat tissues and propylene carbonate. Because this solvent is essentially immiscible with tissues, this method did not require a filtration step.

To a first approximation, hydrophobic organic compounds partition between tissues and blood in accordance with the lipid content of each phase (Van der Molen et al., 1996). *n*-Octanol is frequently used as a surrogate for biological lipid. By assuming a correspondence between *n*-octanol–water partitioning and lipid–water partitioning, Nichols et al. (1991) developed an algorithm to predict blood–water partitioning in rainbow trout. A similar approach, expanded to include partitioning to phospholipid, was used to predict tissue–blood partition coefficients in rats (Poulin and Krishnan, 1995).

Chemical partitioning in a vegetable oil/water system has also been used as a basis for predicting tissue–blood partitioning (Poulin and Krishnan, 1995, 1996a). Alternatively, measured partitioning values can be used to fit the value of slope and intercept terms that relate the extent of partitioning to chemical log K_{ow} and tissue lipid content. Using this approach, tissue-specific equations have been developed for rats, humans, and fish (Bertelsen et al., 1998; DeJongh et al., 1997). An interesting feature of these studies is that fitted slope and intercept terms have suggested that nonlipid cellular constituents contribute substantially to chemical partitioning in lean tissues, including blood.

High-Affinity Binding

High-affinity binding can affect chemical disposition by reducing the fraction of a compound in blood or tissues that is free to diffuse across cellular membranes. An example of high-affinity binding is provided by mammalian PBTK models for TCDD. Although differing in detail, all such models feature some type of specific binding in the liver, generally including an inducible component (Leung et al., 1990). An important outcome of this binding is that TCDD can induce its own redistribution from fat to liver. Fish do not appear to possess these hepatic binding proteins in quantities sufficient to influence TCDD distribution (Nichols et al., 1998). A second example of high-affinity binding is that exhibited toward many heavy metals. Endogenous metal-binding proteins regulate internal concentrations of free metal, both as a means of limiting toxicity and because many metals perform vital biological functions as cofactors and components of enzymes and oxygen transport proteins (Roesijadi and Robinson, 1994). To date, no PBTK models have been developed for metals in fish, and only a few exist for mammals (Gray, 1995; O'Flaherty, 1991, 1996). It may be anticipated that knowledge of metal-binding systems will be required to develop PBTK models for uptake and disposition of most metals.

Urinary and Biliary Elimination

Radiolabeled microspheres have been used to estimate arterial blood flow to the kidneys of arctic grayling (Cameron, 1975), rainbow trout (Barron et al., 1987a), and channel catfish (Schultz et al., 1999). Arterial renal blood flow in the grayling was about 6% of cardiac output; in trout, arterial blood flow ranged from 6 to 10% of cardiac output, depending on the acclimation temperature. Added together, arterial blood flows to the head and trunk kidneys of channel catfish constituted about 9% of cardiac output. Renal portal blood flows in fish are poorly known and cannot be determined using the microsphere method. In most species, however, renal portal blood is supplied largely by the caudal vein, which drains both the skin and the trunk musculature (Satchell, 1992; Smith and Bell, 1975). An estimate of total blood flow to the kidney may therefore be determined by summing the arterial flow and the estimated flow in the caudal vein. In rainbow trout, this results in a total estimated flow equal to about 42% of cardiac output (Nichols et al., 1990). Reported urine flows in fish were summarized by Hickman and Trump (1969) and Hunn (1982). Urine flows in freshwater fish generally range from 1.0 to 10.0 mL hr^{-1} kg^{-1}, while those in saltwater fish range from 0.1 to 1.0 mL hr^{-1} kg^{-1}. Glomerular filtration rates in fish, summarized by Hickman and Trump (1969), usually exceed urine flow in a given species by a factor of about 1.5 to 5.0.

Measured bile flow rates in fish are generally quite low, ranging from 30 to 200 μL hr^{-1} kg^{-1} (Boyer et al., 1976a,b,c; Gingerich et al., 1977; Sanz et al., 1993; Schmidt and Weber, 1973); nevertheless, secretion into bile may represent an important route of elimination for some compounds. Zaharko et al. (1972) incorporated biliary elimination into a PBTK model for methotrexate in stingrays. Bungay et al. (1976) described the biliary elimination of phenol red and its glucuronide conjugate in a PBTK model for the dogfish shark. In both efforts, first-order rate constants were used to represent the sum of metabolic and secretory processes. The value of each rate constant was determined by modeling to measured amounts of chemical in gallbladder bile (because the animals were not fed, all of the bile produced was retained in the bladder). Time constants were incorporated into both models to simulate observed delays in chemical appearance due to the time required to transit the biliary tree. The model presented by Bungay et al. (1976) also incorporated a first-order renal clearance constant to describe the appearance of phenol red and its glucuronide conjugate in urine. Figure 3.29 shows model simulations and measured chemical concentrations in bile and urine given in this study.

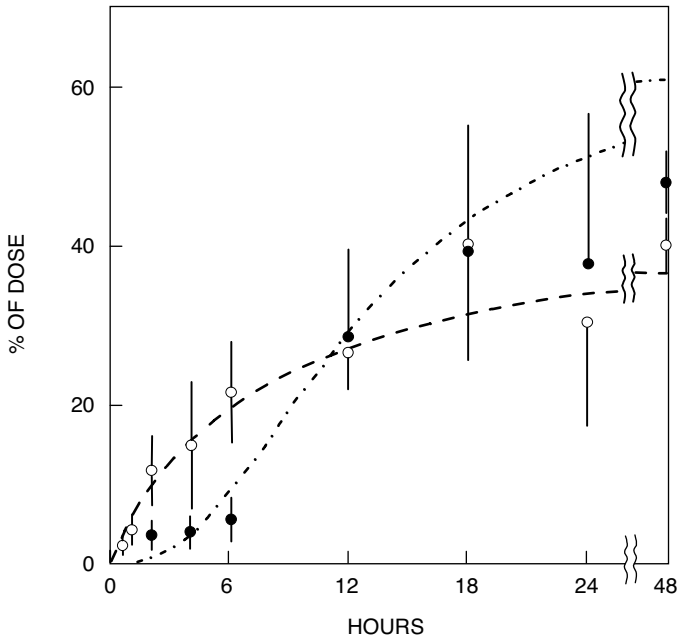

FIGURE 3.29 Time course of phenol red accumulation in the urine (dashed line, open circles) and bile (dot-dashed line, solid circles) of dogfish sharks following intravenous administration. Lines represent simulations generated using a physiologically based toxicokinetic model, and the symbols show measured values. (Adapted from Bungay, P.M. et al., *J. Pharmacokinet. Biopharmaceut.*, 4, 377–388, 1976.)

Biotransformation

Currently, there is considerable interest in using *in vitro* metabolism information to develop PBTK models for compounds that undergo biotransformation. Questions remain, however, concerning the ability of *in vitro* systems to predict *in vivo* metabolism. Due to its known importance as a metabolizing organ, most of the metabolism research conducted to date has focused on the liver. The preparations most commonly used for *in vitro* liver metabolism research are microsomes, freshly isolated hepatocytes, S9 fractions, and precision-cut liver slices. The activity of a microsomal or S9 preparation is commonly expressed as µmoles (of product) min^{-1} mg $protein^{-1}$; that of an isolated hepatocyte preparation is generally expressed as µmoles min^{-1} M $cells^{-1}$ (M = 10^6); and that of a liver slice may be expressed as µmoles min^{-1} $slice^{-1}$, µmoles min^{-1} mg $tissue^{-1}$, or µmoles min^{-1} M $cells^{-1}$.

An approach for incorporating *in vitro* metabolism data into PBTK models was presented by Houston and Carlile (1997a). Briefly, their approach is to: (1) estimate *in vitro* intrinsic clearance ($CL_{in\,vitro,int}$) from the ratio of V_{max}/K_m, determined under linear conditions with respect to time and the amount of enzyme present (i.e., mg of protein or number of cells) (Rane et al., 1977); (2) extrapolate $CL_{in\,vitro,int}$ to *in vivo* intrinsic clearance for the whole liver ($CL_{in\,vivo,int}$) using biologically based proportionality constants; (3) incorporate $CL_{in\,vivo,int}$ into an appropriate liver model that explicitly accounts for both blood-flow limitations and chemical binding relationships; and (4) account for any nonhepatic routes of chemical clearance. A conceptually similar approach was used by Ploemen et al. (1997) to incorporate human *in vitro* data into a PBTK model for ethylene dibromide.

In a review of earlier work involving several mammalian species, Wilkinson (1987) found that hepatic extraction ratios were generally well predicted by *in vitro* estimates of V_{max} and K_m, determined using microsomes, S9 fractions, and isolated hepatocytes. Houston and Carlile (1997b) compared $CL_{in\,vivo,int}$ estimates for 35 compounds, obtained by extrapolating data from rat microsomes, hepatocytes, and liver slices, to observed *in vivo* values. Clearance rates predicted by isolated hepatocytes and liver microsomes were highly correlated. In general, however, isolated hepatocytes provided the most accurate predictions of *in vivo* clearance. $CL_{in\,vitro,int}$ values measured using rat liver slices were lower than those obtained

using isolated hepatocytes when expressed in comparable units (per 10^6 cells). Similarly, Worboys et al. (1996a,b) found that $CL_{in\ vitro,int}$ values for several compounds (expressed as V_{max}/K_m on a whole-liver basis) calculated using data from liver slices were lower than those obtained using isolated hepatocytes. Moreover, the extent of this difference increased with increasing metabolic activity. These studies suggest that slice metabolism rates are influenced by the rate of chemical diffusion from the culture medium into the center of the slice.

Working with rainbow trout, Cravedi et al. (1999) found that isolated hepatocytes were well suited for *in vitro* studies of biotransformation but in some cases failed to produce metabolites found *in vivo* in urine and bile. Research with mammals suggests that it is common for chemicals to be transformed by more than one metabolic reaction. Each of these reactions may exhibit saturable kinetics, characterized by a different set rate and affinity constants. Under these circumstances, it is possible for one pathway to predominate at one substrate concentration while another predominates at higher or lower concentrations.

In several studies with mammals, *in vitro* metabolic parameters have been evaluated by comparison with *in vivo* parameters determined using PBTK models. This approach is based on the assumption that a PBTK model is "correct" with respect to all nonmetabolic aspects of parent chemical disposition. The model can then be used to fit *in vivo* metabolism parameters by simulating the disappearance of parent compound or, less frequently, the appearance of metabolites. Depuration data are generally preferred for this purpose because of the absence of complications associated with chemical uptake and the rapid phase of internal distribution. Not surprisingly, the agreement between *in vitro* metabolism data and fitted *in vivo* parameters has been variable (Fiserova-Bergerova, 1995). In addition to limitations of the *in vitro* systems themselves, substantial metabolism may occur in tissues other than those to which the metabolism was ascribed in the model (and for which *in vitro* data are available). For this reason, fitted *in vivo* parameters are best thought of as *apparent* values representing the summed activities of all relevant enzyme systems and tissues. Law et al. (1991) used K_m and V_{max} data from a trout hepatocyte system to develop a PBTK model for pyrene. Total pyrene clearance greatly exceeded that predicted by *in vitro* metabolism, requiring the incorporation of a fitted clearance constant.

Metabolic parameters determined from *in vivo* studies can also be incorporated into PBTK models. Several authors have attempted to characterize *in vivo* metabolism by measuring products retained by fish or eliminated in bile, urine, feces, and exposure water (Bradbury et al., 1986, 1993; Cravedi et al., 1999; McKim et al., 1986; Stehly and Hayton, 1989a). In practice, however, these measurements are very difficult to make due to low metabolite concentrations and incomplete extraction of samples. One approach to dealing with these problems is to employ a technique called *microdialysis* to measure metabolite concentrations in blood and tissues. McKim et al. (1993) implanted a microdialysis probe into the dorsal aorta of rainbow trout and measured phenol and its major phase II metabolites (phenylglucuronide and phenylsulfate) in the blood of fish exposed to phenol in water. More recently, Solem et al. (2003) used a microdialysis probe to deliver a parent compound (phenol) to the liver in rainbow trout and measure the production of phase I metabolic products (hydroquinone and catechol). An important advantage of microdialysis sampling method is that dialysate samples are free of protein and can be analyzed without extraction. These experiments provide qualitative information about the identity and relative concentrations of metabolic products and can be performed without the need to expose whole fish. Improved *in vivo* calibration procedures and a knowledge of chemical concentration gradients around the microdialysis probe are required, however, before this information can be used to estimate metabolic rate constants.

Utility of Physiologically Based Fish Models

In the true sense of the word, a PBTK model cannot be said to be valid, only that it does or does not reproduce observed kinetics. Confidence in the specification of physiological parameters for a single species is gained if the same set of inputs provides acceptable simulations for several compounds exhibiting diverse partitioning behavior. Similarly, confidence in model structure derives from the ability to simulate the kinetic behavior of the one or more chemicals in several different species by making appropriate changes in physiological inputs.

The principal advantage of a PBTK model is that it provides descriptions of the chemical concentration time course in specific tissues of interest. This provides a direct link to studies of toxic effect and in particular to observations for a specific site of action. As an example, numerous studies have suggested that TCDD is highly toxic to fish in early life stages. In wild fish, this exposure occurs following maternal transfer of accumulated residues to the developing ovaries. A PBTK model for the maternal transfer of TCDD was therefore developed to support studies of TCDD embryotoxicity in brook trout (Nichols et al., 1998). A second important use of PBTK models is to evaluate competing assumptions about factors that control chemical uptake and disposition. Nichols et al. (2004a,b), for example, used a PBTK model to investigate factors that control dietary uptake of hydrophobic organic compounds. Based on these studies, it was concluded that a log K_{ow}-dependent kinetic limitation prevents the gut tissues and contents from attaining an internal equilibrium. The nature of this limitation remains unknown, but it does not appear to be related to diffusion across the gastrointestinal epithelium. PBTK models can also be used to estimate important kinetic parameters that may be difficult or impossible to determine otherwise. Several examples of this approach appear in the preceding text, including the use of models to solve for biliary elimination rate constants (Bungay et al., 1976; Zaharko et al., 1972), dermal permeability constants (Nichols et al., 1996), and metabolic rate constants (Law et al., 1991).

The utility of PBTK models for fish was demonstrated particularly well by a linked toxicokinetic and toxicodynamic model for paraoxon in rainbow trout (Abbas and Hayton, 1997). Paraoxon is produced in mammals by oxidative metabolism of the insecticide parathion and is a potent inhibitor of acetylcholinesterase (AChE) and carboxylesterase (CaE). The activation of parathion to paraoxon is thought to be insignificant in trout; however, paraoxon can be formed in aquatic environments by nonenzymatic conversion of parathion and may be available for uptake by fish directly from water. The toxicokinetic portion of this model accurately simulated the uptake of paraoxon from water and its distribution to selected tissues. This information was then combined with experimentally determined rates of AChE and CaE synthesis and degradation, as well as biomolecular inhibition rate constants determined in previous studies with rodents. The linked model successfully reproduced the observed time course of AChE inhibition in each of the tissues examined and confirmed the role of CaE in detoxification of paraoxon by sequestration of active compound (Figure 3.30).

Another use of PBTK models is to evaluate the potential for variability among individuals of a single species to impact chemical uptake and disposition. A simple approach to this question was provided by Lien et al. (2001), who simulated the kinetics of three chlorinated ethanes in lake trout using physiological data from individual animals. Simulations for each animal were then plotted to represent the range of anticipated outcomes. Alternatively, statistical distributions for individual model inputs can be characterized and used in a repeated sampling (Monte Carlo) design to generate a distribution of predicted outcomes. The principal advantage of the former approach is that it explicitly treats the possible interdependence of model parameters. The Monte Carlo approach can be adapted to deal with parameter interdependence. In practice, however, this is difficult because the necessary information is generally lacking.

Mammalian PBTK modeling efforts are largely driven by the needs of human health risk assessment and in particular by the need to extrapolate data from laboratory test animals to humans. In this context, it is necessary to consider factors such as aging, health status, and dietary habits that could result in increased vulnerability among a subset of the human population. For fish and other ecological receptors, the principal driving force behind PBTK model development is the need to identify species that, for toxicokinetic or other reasons, may be particularly vulnerable. Simultaneous exposure by multiple routes is common in the environment. PBTK modeling is useful in this regard as it enables modeling of multiple chemical fluxes at all relevant exchange surfaces.

The principal disadvantage of using a PBTK modeling approach is that considerable effort is required to develop models for new species and chemicals. Future applications of the PBTK modeling approach will depend on the systematic collection of necessary biological and chemical information, as well as the development of methods for estimating critical parameters when data for a compound and species are limited or absent altogether. Quantitative structure–activity relationship (QSAR) approaches hold particular promise as a means of obtaining tissue partitioning estimates, and perhaps also metabolic rate predictions (McKim and Nichols, 1994; Parham and Portier, 1998; Parham et al., 1997; Poulin and Krishnan, 1996b; Verhaar et al., 1997).

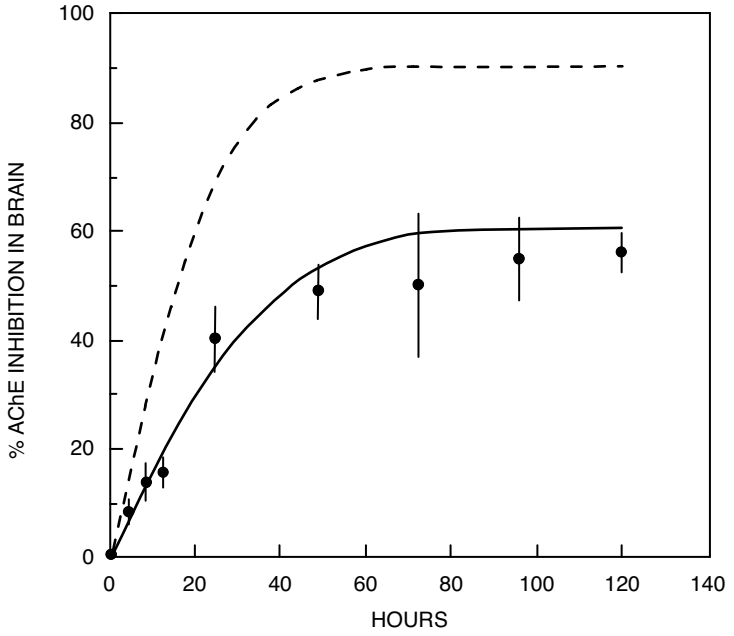

FIGURE 3.30 Metabolism of paraoxon by carboxylesterase (CaE). Paraoxon concentrations were linked to acetylcholinesterase (AChE) inhibition in the brain of rainbow trout using a physiologically based toxicokinetic/toxicodynamic model. Model predictions with and without CaE metabolism are shown as solid and dashed lines, respectively; individual points show measured levels of AChE inhibition. (Adapted from Abbas, R. and Hayton, W.L., *Toxicol. Appl. Pharmacol.*, 145, 192–201, 1997.)

Noncompartmental Analysis

Noncompartmental analysis can be used to estimate of the volume of distribution (V_{ss}), total body clearance (CL), and persistence of a chemical (mean residence time). Because it does not rely on curve-fitting techniques to fit model-based equations to experimental data, this approach avoids a number of statistical considerations such as choice of weighting function, multiple minima in the sum of squares fitting criterion, and large variance in parameter values. Although this approach does not characterize the kinetics of distribution of chemical, as do compartmental and PBTK modeling methods, it can be used to estimate basic pharmacokinetic parameters, even when the data are highly variable. Noncompartmental analysis was used by Barron et al. (1987b) to investigate the temperature dependence of di-2-ethylhexylphthalate kinetics in rainbow trout. Total body clearance values calculated in this manner were very close to those estimated using a compartmental model. Alavi et al. (1993) used noncompartmental analysis to estimate pharmacokinetic parameters for sulfachlorpyridazine in channel catfish. To date, however, very few researchers have used noncompartmental methods in kinetic studies with fish. The application of noncompartmental analysis to bolus dosing data is described below. Modifications of this approach are required to deal with more complex dosing regimens.

Intravascular Bolus Administration

No explicit model structure is used in noncompartmental analysis; however, the chemical must be measured in and exit the body from the pool or compartment of its introduction (Matis et al., 1985). The method is based on the concept of mean residence time (MRT) of the chemical in the body. The MRT is the average time that all the molecules of chemical spend in the body after their simultaneous administration; for example, if the dose consisted of 100 chemical molecules administered as a bolus, then the MRT would be the sum of the individual residence times of each chemical molecule divided by 100. Following bolus administration, the rate of chemical elimination at any time is:

$$dX/dt = -CLC_p \tag{3.114}$$

where X represents the amount of chemical in the body, CL is the plasma clearance of the chemical, and C_p is the plasma concentration. From this relationship, the amount of chemical eliminated over a small time interval, dt, is:

$$dX = -CLC_p dt \tag{3.115}$$

At the time (t) that dX is eliminated, dX has spent t time in the body. The total time in residence for all the molecules that make up X is the sum of the products of all the dX multiplied by the corresponding residence times; that is, the integral of tdX:

$$\int_0^\infty -CLC_p t\,dt \tag{3.116}$$

The total amount introduced into the body is the total amount eliminated, which may be expressed as:

$$\int_0^\infty -CLC_p dt \tag{3.117}$$

The mean residence time is the total time in residence divided by the total amount introduced into the body:

$$MRT = \frac{\int_0^\infty -CLC_p t\,dt}{\int_0^\infty -CLC_p\,dt} = \frac{\int_0^\infty C_p t\,dt}{\int_0^\infty C_p dt} = \frac{AUMC}{AUC} \tag{3.118}$$

where AUC is the area under the plasma concentration–time profile, and AUMC is the area under the moment curve, which is the integral of the product of plasma concentration and time.

After intravascular bolus administration of chemical, the MRT can be used to calculate the V_{ss} using the relationship:

$$V_{ss} = MRT\ CL \tag{3.119}$$

where:

$$CL = dose/AUC \tag{3.120}$$

Even when the plasma concentration–time profile is complex and irregular, it is generally possible to calculate AUC and AUMC, provided the profile is completely characterized.

To obtain the total AUC, the area beyond the last measured plasma concentration must be estimated. This may be accomplished using the relationship:

$$AUC_{t,last-\infty} = C_{p,last}/\lambda_z \tag{3.121}$$

where λ_z is –2.3 times the slope of the log-linear portion. Although the plasma concentration–time profile can be highly irregular, it has to have a terminal log-linear phase to provide a λ_z value for the extrapolation. In the absence of a log-linear phase, it is necessary to have a sufficient number of samples so the measured concentrations fully outline the AUC. In that case, a CL value may be obtained but a value for V_{ss} cannot be determined.

As a practical matter, it is important that the extrapolated portion of the AUC be relatively small (ideally, less than 10% of the total AUC). Due to the fact that the extrapolated part is just that, there is some uncertainty in its true value, as the log-linear phase may not continue on the extrapolated line.

Calculating the AUMC also involves determination of that part of the total area that lies beyond the last measured plasma concentration. If a log-linear phase is present:

$$AUMC_{t,last-\infty} = \frac{t_{last}C_{p,last}}{\lambda_z} + \frac{C_{p,last}}{\lambda_z^2} \tag{3.122}$$

and AUMC is then $AUMC_{0-t,last} + AUMC_{t,last-\infty}$. The problem of extrapolation is larger for AUMC than for AUC because much more of the C_p,t profile has been defined by the measured concentrations than has been defined by the C_{pt},t profile.

Bioconcentration, Bioaccumulation, and Biomagnification

In this section, chemical bioconcentration, bioaccumulation, and biomagnification in fish are treated as special topics because of their importance in the context of current regulatory approaches. By convention, the term *bioconcentration* refers to the accumulation of waterborne chemicals by aquatic animals through nondietary routes (Veith et al., 1979). The importance of bioconcentration as a measure of chemical accumulation by fish has been recognized since the 1960s (Hamelink et al., 1971). By the, 1970s, however, it had become apparent that uptake of very hydrophobic organic compounds by fish within an environmental setting was dominated by the dietary route of exposure (Bruggeman et al., 1981, 1984). The term *bioaccumulation* refers to the accumulation of chemicals by all possible routes of exposure. As described below, bioconcentration and bioaccumulation are expressed by referencing the chemical concentration in a fish to that in water or sediment. The term *biomagnification* refers to a stepwise increase in chemical concentration in organisms representing successively higher trophic levels, resulting from the ingestion of contaminated organisms at lower trophic levels. Biomagnification is expressed, therefore, by referencing the extent of chemical bioaccumulation in a predator to that of its prey.

Processes that control the rate and extent of chemical accumulation in fish were described in earlier sections of this chapter. Thus, uptake directly from water is dependent on factors that control chemical flux across the gills and skin, including the bioavailability of waterborne compounds, limitations on uptake imposed by water and blood flows, and the relative affinity of chemicals for blood, skin, and water. Similarly, the rate of uptake from dietary sources is dependent on the oral bioavailability of ingested compounds and the extent to which these compounds become concentrated in prey items. Elimination of parent compounds may occur at the gills, skin, and gut or by secretion into bile or urine.

Chemical accumulation may be substantially reduced by biotransformation. In controlled exposures with rainbow trout, Barron et al. (1989) found that measured concentrations of the phthalic acid ester di-2-ethylhexylphthalate were 100 to 1000 times lower than those predicted from chemical hydrophobicity. Similar findings have been reported for several azaarenes (Southworth et al., 1980) and polycyclic aromatic hydrocarbons (PAHs) (Jonsson et al., 2004). Experimental inhibition of metabolism resulted in an increase in bioconcentration of pentachlorophenol (Stehly and Hayton, 1989a).

In field sampling efforts, low levels of bioaccumulation in fish have been found for hydrophobic but easily metabolized PAHs (Varanasi et al., 1989). Lower than expected levels of bioaccumulation for some lower chlorinated dibenzo-*p*-dioxins and dibenzo-*p*-furans in field-caught fish were also attributed to metabolism (Opperhuizen and Sijm, 1990). Care is required, however, when interpreting field sampling data because metabolism may occur at multiple sites within a food web, altering the concentration of chemical to which a fish is exposed.

In Chapter 14, Mackay and Milford describe a chemical fate and transport model and show how this model can be used to predict chemical concentrations in a water–sediment system. Outputs from such models are often used as inputs to models of chemical bioconcentration and bioaccumulation in fish. Models of bioconcentration and bioaccumulation take different forms, depending on simplifying assumptions and the need to mechanistically describe controlling processes. In regulatory applications, it is

common to start with the simplifying assumption that all elements of an ecosystem are at steady state. Non-steady-state models may be required, however, when chemical loadings to a system change over time or when other processes result in a substantial disequilibrium among water, sediment, and biotic components.

Techniques used to measure, express, model, and predict bioconcentration and bioaccumulation are described below. The concepts described in these sections are then combined in a simple food web. A brief description of the fugacity-based approach to chemical modeling is given to provide a basis for comparison with other modeling approaches. A more detailed description of the fugacity-based modeling approach is given in Chapter 14.

Bioconcentration

The term used to quantify the magnitude of bioconcentration in aquatic systems is the *bioconcentration factor* (BCF), which is defined as a proportionality constant relating the chemical concentration in a fish (or a portion thereof) to that in water under steady-state conditions. It is a measure of the propensity of a chemical to accumulate in fish, and BCF values fall between 0 and infinity (Veith et al., 1979). BCFs have units of water volume/tissue weight (e.g., mL/g) and can be viewed conceptually as the water volume containing the amount of chemical concentrated in 1 gram of animal tissue (Barron, 1990). BCFs can be measured directly by exposing fish in water until steady state is attained or estimated using simple kinetic models in conjunction with short-term uptake and elimination studies. Using these procedures, BCFs have been determined for a large number of nonionic organic compounds, as well as some metals, metalloids, and organometallics. Several authors have used these datasets to develop QSARs that relate the BCF of a compound to one or more physicochemical properties. These QSAR models are used extensively to estimate BCFs for untested chemicals and by extension to predict their behavior in aquatic systems.

As indicated previously, hydrophobic compounds tend to partition into tissue lipids. BCFs for such compounds may be interpreted, therefore, in terms of a chemical distribution between fish lipid and water. When making these interpretations, it is important to distinguish between a steady-state chemical distribution and true thermodynamic equilibrium. A steady-state condition is defined as an unchanging chemical concentration, and a thermodynamic equilibrium is a minimum energy state that reflects the relative affinity of a chemical for two partitioning phases. A system at steady state is unlikely to be in thermodynamic equilibrium in the presence of dynamic processes such as biotransformation and growth.

The lipid content of fish varies widely. Variability among BCFs for the same or similar compounds in more than one species or life stage can be reduced, therefore, by normalizing for differences in lipid content, although factors such as lipid composition may also be important (Ewald and Larsson, 1994; Vigano et al., 1992). The lipid-normalized BCF has units of mL/g lipid.

Steady-State Exposures

Steady-state exposures are conducted by exposing fish to a constant concentration of chemical in water for an extended period of time (U.S. EPA, 1996). Typically, these studies are conducted by exposing small fish in a flow-through system for 28 days. The fish are serially sampled, and whole-body concentrations of the chemical are quantified. This method may underestimate the true steady-state BCF if exposure times are too short. The length of time required to reach steady state depends on uptake and elimination rates; for example, specific PCB congeners with elimination half-lives of 100 days or longer may require 1 year to reach 90% of steady state (Barron et al., 1994). Steady-state exposures are species or site specific and allow for the incorporation of environmentally realistic exposure conditions.

Kinetic Modeling

Bioconcentration factors may also be estimated by applying simple kinetic models to uptake and elimination datasets (Barron et al., 1990; Branson et al., 1975; Landrum et al., 1992; U.S. EPA, 1996). Uptake from water is generally assumed to be first order with respect to the chemical concentration in

water (C_w), and elimination is assumed to be first order with respect to chemical concentration in the fish (C_f). Assuming further that the fish can be adequately represented as a single compartment, a mass balance on this system may be written as:

$$dC_f/dt = k_1C_w - k_2C_f \tag{3.123}$$

where k_1 and k_2 are uptake and elimination rate constants, respectively, with units of inverse time. By assuming that the specific gravity of fish tissue is approximately 1.0, k_1 can also be expressed as a clearance rate, normalized for body weight (e.g., mL water per g tissue per day). The value of k_1 can then be interpreted by comparing it to physiological measures such as ventilation volume.

At steady state, $k_1C_w = k_2C_f$. Because, by definition, the BCF equals C_f/C_w, rearrangement of this equation gives the relationship used to estimate the BCF from kinetic data:

$$BCF = k_1/k_2 \tag{3.124}$$

Elimination rate constants are typically estimated from the slope of log-transformed depuration data. Uptake rate constants may then be estimated by fitting uptake data to Equation 3.123 using nonlinear regression methods. Kinetic models have the advantage of being chemical and species specific and do not require exposure to steady state. The BCF of a chemical may be unreliably estimated by this method if the duration or extent of sampling of the elimination phase is inadequate or if an inappropriate model is used (Stehly and Hayton, 1989b).

QSAR Models

Empirically based QSAR models are used to estimate BCFs for untested compounds based on their physicochemical properties. Most are linear regression models relating the log of the BCF of a compound to the log of its octanol–water partition coefficient (Lipnick, 1995). Veith et al. (1979), for example, developed the following relationship from BCFs in fish for chemicals with log K_{ow} values ranging from 1 to 7:

$$\log BCF = \left(0.85 \times \log K_{ow}\right) - 0.7 \tag{3.125}$$

QSAR models of this type have generally been developed using data for halogenated organic chemicals (Mackay, 1982). Implicit in this approach are the assumptions that: (1) an octanol–water system is an appropriate surrogate for a fish lipid–water system, and (2) bioconcentration results from thermodynamically driven partitioning between water and the lipid phase of the fish (Hansch et al., 1989). QSAR models can also be fit to lipid-normalized BCF data. Correspondence with the lipid-partitioning assumption is indicated when lipid-normalized log BCF values are approximately equal to log K_{ow} (Briggs, 1981). Additional QSAR models have been developed based on water solubility and molecular size descriptors (Hawker, 1990; Isnard and Lambert, 1988; Schuurmann and Klein, 1988).

Bioaccumulation

Bioaccumulation Referenced to Water

Bioaccumulation of contaminant residues in fish may be referenced to the chemical concentration in water using a bioaccumulation factor (BAF). BAFs are generally normalized to a fish's lipid content to permit comparisons among species and between predatory fish and their prey (to assess biomagnification; see below). For very hydrophobic compounds, adjustments to the total chemical concentration in water may be attempted as a means of accounting for reductions in bioavailability due to binding to dissolved and particulate organic carbon (Burkhard et al., 2003). Because the free chemical concentration in water may be difficult to measure, these adjustments are generally based on empirically derived relationships (Chin and Gschwend, 1992). The units obtained from this approach are ng/kg lipid divided by ng freely dissolved contaminant per liter of water.

Mechanism of Biomagnification

Field sampling efforts suggest that in some cases measured BAFs in fish exceed the BCF that would have been expected from an equilibrium distribution of chemical between the fish and water. These observations have been attributed to processes that accompany the digestion of contaminated food items. According to the *digestion hypothesis*, absorption of dietary lipid and reductions in meal volume increase chemical activity in the gut contents above that of the ingested meal, resulting in an inwardly directed diffusion gradient and a potential for biomagnification of chemical residues (Connolly and Peterson, 1988; Gobas et al., 1993a,b, 1999). Under these circumstances, the gills become a route of net chemical elimination and not uptake, and the extent of bioaccumulation and biomagnification is determined by the balance between chemical uptake within the gut, branchial efflux, biotransformation, and growth.

The extent of biomagnification is defined using a biomagnification factor (BMF), which is the lipid-normalized chemical concentration in a predator divided by that of its prey. For a given trophic transfer step, a compound is said to *biomagnify* when the BMF is greater than 1. Within a simple food chain, BMFs for each trophic transfer step can be multiplied. Under these circumstances, the BAF for fish that occupy the highest trophic level will exceed the partitioning-based BCF prediction by an amount equal to the product of BMFs for all relevant trophic transfers.

Support for the digestion hypothesis has been obtained in studies with several fish species. In feeding studies with guppies and goldfish, the ratio of feces to food fugacity increased with chemical log K_{ow}, attaining a maximum value of 4.6 (in guppies) for the pesticide mirex (as described below, fugacity is a measure of chemical activity; see Gobas et al., 1993a). The ratio of intestinal contents to food fugacity in a natural population of white bass (*Morone chrysops*) also increased with chemical log K_{ow}, attaining a value of about 2.2 for 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (Russell et al., 1995). In rainbow trout and rock bass (*Ambloplites rupestris*) exposed to 2,2′,4,4′,6,6′-hexachlorobiphenyl, the chemical fugacity in chyme following uptake of dietary lipid exceeded that of food by a factor of 7 to 8 under both laboratory and field conditions (Gobas et al., 1999).

Nichols et al. (2004a) developed a PBTK model for the dietary uptake of hydrophobic chemicals by fish that accounts for the absorption of dietary lipid and reductions in meal volume. The model was then used to simulate chronic exposures to a set of hypothetical high log K_{ow} compounds (Nichols et al., 2004b). The results of this effort showed that a log K_{ow}-dependent diffusion resistance acts along the entire length of the GIT to limit dietary uptake of high log K_{ow} compounds. This decrease in diffusive uptake appears to be responsible for a log K_{ow}-dependent decrease in absorption efficiencies, BAFs, and BMFs at log K_{ow} values greater than about 6 (Figure 3.9). Metabolism and growth were predicted by the model to result in lower BMFs at all log K_{ow} values. In either case, however, BMFs continued to increase with chemical log K_{ow} (assuming constant diffusion resistance). In field sampling efforts, BAFs for persistent organochlorines in fish have been shown to increase with log K_{ow} up to a log K_{ow} value of 6 or 7 and then level off or decline at higher log K_{ow} values (Burkhard, 1998; Thomann, 1989).

Bioaccumulation Referenced to Sediment

Aquatic sediments are formed from the deposition of particles and colloids and can act as both a sink and source of contaminants. Long-term contaminant input can lead to sediment concentrations that exceed the water concentration by several orders of magnitude. Both metals and hydrophobic organic compounds bind to sediments, but the nature of these interactions differs. Under reducing conditions, metals tend to form insoluble complexes with sulfide, and the acid volatile sulfide (AVS) content of sediment has been used to normalize for differences in apparent toxicity of some sediment-associated metals (Carlson et al., 1991; Di Toro et al., 1990). In oxic sediments, however, organic material may provide the major site for metal binding (Fu et al., 1992). Other factors that may influence metal bioaccumulation from sediment include: (1) speciation, (2) transformation to organic derivatives, (3) interactions of different metals, and (4) sediment chemistry (salinity, iron oxide content, redox potential, and pH) (Bryan and Langston, 1992).

The binding of organic contaminants to sediments has been related to the organic carbon content, clay type and content, cation exchange capacity, pH, and particle surface area of the sediment (Knezovich et al., 1987). In many systems, the organic carbon content of sediment (typically 0.5 to 3% of sediment mass) predicts contaminant toxicity (Bierman, 1990; Di Toro et al., 1991). Inferred by these observations

is an association between toxicity and bioavailability. The source of this organic carbon (e.g., mud, plant material) may influence bioavailability and bioaccumulation (DeWitt et al., 1992). In some regions, soot carbon from the combustion of fossil fuels may also contribute to sediment binding of organic compounds (Accardi-Dey and Gschwend, 2003; Persson et al., 2002). Although generally present in small amounts (a few percentage points of total carbon), the affinity of soot carbon for some hydrophobic compounds may be considerably higher than that of organically derived carbon.

The extent of chemical bioaccumulation from sediment is typically expressed using either a bioaccumulation factor or a biota-to-sediment accumulation factor (BSAF) (Spacie et al., 1995). The sediment BAF is the ratio of the chemical concentration in fish to chemical concentration in sediment. Sediment BAFs have units of sediment mass per unit of tissue weight (wet weight/wet weight) and can be viewed conceptually as the sediment mass containing the amount of chemical concentrated in 1 gram of fish tissue. The BSAF is the ratio of the lipid-normalized chemical concentration (ng chemical per g lipid) in fish to the organic carbon normalized concentration in sediment (ng chemical per g organic carbon). Thorsen et al. (2004) adjusted this ratio further to account for chemical binding to soot carbon in sediments and determined that this binding was unlikely to have a major impact on BSAFs for PAHs in two freshwater bivalves. Additional work is required to determine whether such adjustments are needed for other compounds.

Biota-to-sediment accumulation factors are used to reduce variability in measured bioaccumulation due to variation in organism lipid content and sediment organic carbon concentrations. BSAFs in fish typically range from 0.1 to 10 (Bierman, 1990; Burkhard et al., 2004; Rubinstein et al., 1984; Schuytema et al., 1988). Values greater than 1 are often interpreted as evidence for biomagnification of chemical residues coupled with little or no biotransformation. Values less than 1 have been attributed to biotransformation, although other factors such as growth and low dietary assimilation efficiency may contribute.

Methods for estimating the bioaccumulation of sediment-associated contaminants include steady-state exposures, equilibrium partitioning (EqP) theory, and kinetic models. Each approach has been used extensively to estimate BSAFs for benthic invertebrates. Chemical accumulation by benthic invertebrates is important because consumption of these organisms by fish provides a route for translocation of sediment-associated contaminants to aquatic food webs. Direct exposures also have been employed to study the bioaccumulation of sediment contaminants by fish that live in intimate contact with these sediments (Hellou et al., 1995; Payne et al., 1995). BSAFs for high trophic level fish are generally predicted using a food web model that includes a benthic component (see below).

In each case, it should be noted that the BSAF definition makes no assumptions about a chemical equilibrium between the organism and sediment. Although it may be reasonable to assume that an equilibrium exists between sediments and benthic invertebrates, this assumption becomes more problematic for high-trophic-level fish. Field-derived BSAFs must be interpreted, therefore, in the context of changes in toxicant loadings to the environment, as well as other potential sources of disequilibrium.

Equilibrium Partitioning Theory of Bioaccumulation from Sediments

The equilibrium partitioning (EqP) theory of bioaccumulation from sediments is based on the assumption that chemicals partition between sediment, pore water, and aquatic organisms in accordance with thermodynamic principles (Di Toro et al., 1991). The theory predicts that the equilibrium concentration of a chemical in sediment pore water (C_w) is controlled by the concentration of the chemical in sediment (C_s), the organic carbon–water partition coefficient (K_{oc}) of the chemical, and the fraction of organic carbon (OC_s) in sediment according to:

$$C_w = C_s / \left(K_{oc} OC_s \right) \tag{3.126}$$

Bioaccumulation of contaminants by sediment-dwelling biota (C_{biota}) is determined by the lipid content of the organism (L_{biota}), C_s, and OC_s:

$$C_{biota} = L_{biota} \left(C_s / OC_s \right) \tag{3.127}$$

Substituting Equation 3.126 into Equation 3.127 and adopting the assumption that $K_{ow} = K_{oc}$ gives the relationship:

$$C_{biota} = L_{biota}K_{ow}C_w \tag{3.128}$$

This equation suggests that the contaminant concentration in the organism can be estimated from the concentration freely dissolved in pore water. Hellou et al. (1995) suggested that K_{ow} and K_{oc} are not equal but are instead related by a proportionality constant. A modification of Equation 3.128 was derived by Hellou et al. (1995) by assuming that $K_{ow} = 0.4K_{oc}$ and that the density of sediment is 1.6 kg/L:

$$BSAF = L_{biota}/\left(OC_s \times 0.64\right) \tag{3.129}$$

Assumptions of the EqP theory include concentration-independent uptake and an absence of biotransformation or degradation of the contaminant. An implicit assumption of the EqP theory is that the bioavailable concentration in water is the freely dissolved portion; the presence of DOC does not, therefore, affect equilibrium partitioning. According to EqP theory, the equilibrium level accumulated by an organism is independent of the number and types of exposure routes (e.g., sediment ingestion or pore water exposure).

An extensive review of data supporting the EqP theory was given by Di Toro et al. (1991), who concluded that sediment-to-sediment variation in bioavailability (assessed by toxicity) can be reduced by a factor of two or three by application of EqP theory and that particle size effects are minimal. Using the principles of EqP theory, the U.S. Environmental Protection Agency has developed procedures to derive equilibrium partitioning sediment benchmarks (ESBs) for the protection of benthic organisms from adverse effects due to nonionic organic compounds (U.S. EPA, 2003).

Kinetic Models for Chemical Accumulation from Sediment

Kinetic models of contaminant bioaccumulation from sediment have been primarily developed for benthic invertebrates, including amphipods, insects, and mollusks (Lee, 1992). The simplest model is:

$$BAF = k_s/k_{el} \tag{3.130}$$

where k_s is the sediment uptake constant (g sediment/[g tissue × time]), expressed as a clearance term, and k_{el} is the elimination rate constant (1/time). This model is analogous to that given previously (Equation 3.124) to describe chemical bioconcentration from water. According to the model, sediment BAFs are independent of the chemical concentration in sediment but increase with any factor that increases the uptake rate constant or decreases the elimination rate constant.

Food Web Models of Bioaccumulation in a Sediment–Water System

Food web bioaccumulation models describe the contaminant mass balance in biota that comprise an aquatic food web (Thomann et al., 1992b). Contaminant concentrations in biota are calculated using mathematical equations that describe the dominant uptake and elimination processes. These processes may include equilibrium partitioning (e.g., sediments to benthos, water to plankton), chemical uptake from water, ingestion of contaminated food, growth, and excretion. Contaminant concentrations in source compartments (water and sediments) are generally assumed to have a homogeneous distribution and to be in steady-state equilibrium with biota comprising the lowest level of the food web.

Food web models are particularly useful for compounds that bioaccumulate in plankton and benthic invertebrates and then biomagnify in fish through successive trophic transfers. These models are used extensively to estimate BAFs, BSAFs, and BMFs and to determine the relative importance of benthic (sediment) and pelagic (water) contaminant sources. In a regulatory setting, these models can be used to characterize a contaminated site and evaluate various remedial options. The utility of this approach is limited, however, by the need to incorporate a large number of parameters, many with high levels of uncertainty. Model parameters typically include body size, temperature, feeding rate, prey selection, lipid content, and dietary bioavailability (absorption efficiency). The proportion of time each trophic group feeds within the contaminated food web may be an additional source of variability and uncertainty.

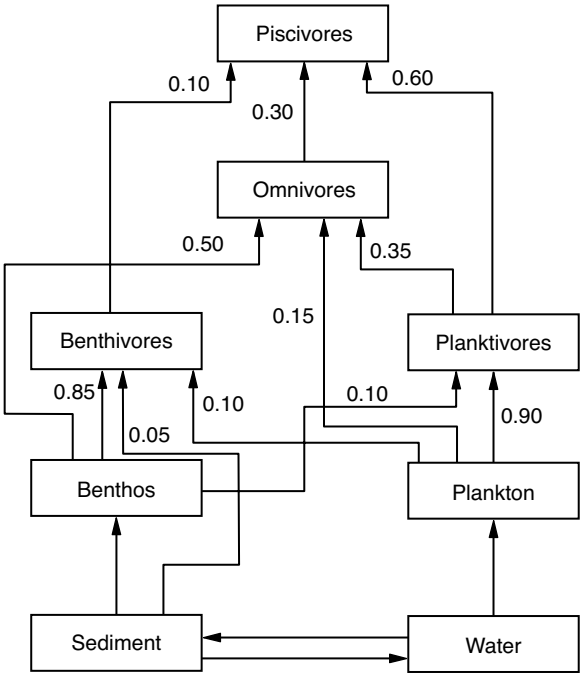

FIGURE 3.31 Schematic representation of a food wed bioaccumulation model. Numbers indicate the fractional contribution of each contaminant source to the diet of biota. Arrows without numbers designate equilibrium partitioning relationships.

The following section summarizes the model-based equations of a generalized food web model adapted from Thomann et al. (1992a) and Gobas (1993). This model can be viewed conceptually as a series of compartments linked by transport pathways (Figure 3.31). Compartments that correspond to biota are based on assumed feeding habits. Sediment and water compartments act as sources of chemical contamination. Generally, contaminant concentrations in sediment and water are specified as input parameters. These concentrations may be based on measured values for a site of interest or obtained as predictions from a fate and transport model (see Chapter 14). In addition to the pathways shown in Figure 3.31, the model also calculates chemical concentrations in fish tissues that result from exposure to a contaminant dissolved in the water column. More complex food web models are available, such as those based on species- and age-specific bioaccumulation (Connolly, 1991).

Plankton and Benthos

Contaminant concentrations in plankton and benthos are calculated from equilibrium partitioning relationships based on organism lipid content and the bioavailable chemical concentration in water and sediment pore water, respectively. For the simplified model in Figure 3.31, contaminant concentrations are assumed to be equal in phytoplankton and zooplankton. This simplification may be reasonable for chemicals such as PCBs that partition rapidly and exhibit little or no biomagnification between phytoplankton and zooplankton (Gobas, 1993; Oliver and Niimi, 1988). The assumption of equilibrium partitioning may depend on the rate of uptake relative to the rate of growth of biomass. Swackhamer and Skoglund (1993) concluded that the establishment of an equilibrium was unlikely under conditions of rapid phytoplankton growth.

Benthivores, Planktivores, Omnivores, and Piscivores

Predator trophic groups are defined by their prey selection. Contaminant concentrations in predator trophic groups (C_f) are calculated as the ratio of chemical intake (from water [k_{uw}] and food [k_{uf}]) to chemical elimination (due to excretion [k_{ex}] and egestion [k_{eg}]) and growth dilution (k_g):

$$C_f = \frac{\left[\left(k_{uw}C_w\right) + \left(k_{uf}\Sigma P_i C_i\right)\right]}{k_{ex} + k_{eg} + k_g} \tag{3.131}$$

where dietary intake is the sum of the proportion of each prey trophic group selected (P_i) multiplied by the contaminant concentration in the dietary item (C_i). The uptake rate from water (k_{uw}) is dependent on the initial transfer of a chemical from water to the aqueous phase of the organism and then to the lipid phase of the organism:

$$k_{uw} = \frac{1}{\left[\left(W_v/Q_w\right) + \left(W_v/K_{ow}Q_l\right)\right]} \tag{3.132}$$

where W_v is the weight of the organism, and Q_w and Q_l are transport rates in the aqueous and lipid phases. Q_w is estimated from an empirical relationship that relates chemical transport to body weight:

$$Q_w = 88.3W_v^{0.6} \tag{3.133}$$

Q_l is less well known than Q_w but is thought to be about 100 times smaller than Q_w:

$$Q_l = Q_w/100 \tag{3.134}$$

The uptake rate from food (k_{uf}) is dependent on dietary absorption efficiency (α, the fraction of the dietary dose that is absorbed by the animal), feeding rate (FR), and W_v:

$$k_{uf} = \alpha FR/W_v \tag{3.135}$$

The FR depends on both allometric and bioenergetic considerations and may be estimated from an empirical relationship based on body weight and ambient temperature (T in °C; annual average):

$$FR = 0.022W_v^{0.85}e^{0.06T} \tag{3.136}$$

The excretion rate of a chemical across the gills (k_{ex}) is dependent on the transfer of chemical from lipid to the aqueous phase of the fish and then to ambient water:

$$k_{ex} = \frac{1}{\left[\left(W_vL_iK_{ow}/Q_w\right) + \left(W_vL_iQ_l\right)\right]} \tag{3.137}$$

The fecal egestion rate (k_{eg}) is calculated as 0.25 times the uptake rate from food:

$$k_{eg} = 0.25k_{uf} \tag{3.138}$$

The growth of a fish over time tends to reduce contaminant concentrations by increasing the tissue mass into which chemicals are distributed. This phenomenon is referred to a *growth dilution* and is accounted for by calculating k_g as a weight-dependent coefficient:

$$k_g = 0.000502W_v^{-0.2} \tag{3.139}$$

Food web models are typically calibrated using existing data on contaminants in an ecological system similar to that which is being modeled. Parameter inputs, including body size, temperature, prey selection, lipid content, and asorption efficiency, may be determined from literature values or site-specific measurements. As with compartmental and PBTK modeling, parameter values can be adjusted to obtain better agreement between observed and model-estimated concentrations of contaminants in biota. As

noted by Thomann et al. (1992b) and others, equilibrium partitioning models can substantially underestimate the accumulation of PCBs, chlorinated pesticides, and other organochlorine compounds in upper trophic level fishes because trophic transfer is not considered.

Fugacity-Based Models

Fugacity-based models were initially developed from engineering principles (models of gas transfer) and have been applied extensively in environmental fate modeling to estimate chemical concentrations in different environmental compartments (phases), including air, water, sediment, and biota. Using a fugacity approach, the concentration of a contaminant is expressed in units of moles/volume (e.g., mol/m^3) rather than mass/mass (e.g., mg/kg body weight) or mass/volume (e.g., mg/L water) and is calculated as the product of chemical fugacity f (Pa) and the fugacity capacity Z (mol/Pa m^3). The fugacity of a compound is an expression of chemical activity that characterizes the escaping tendency from a particular phase, while *fugacity capacity* can be viewed as a kind of solubility (Mackay and Paterson, 1982). In general, fugacity-based models assume that a chemical achieves equilibrium between all of the environmental phases, although transfer resistances (model terms that limit transfer from phase to phase) can be incorporated to model retention of a chemical.

Fugacity-based models have been developed to describe chemical bioaccumulation in fish (Gobas et al., 1989) and trophic transfer in aquatic food webs (Campfens and Mackay, 1997). To illustrate this approach we can consider the one-compartment model for chemical bioconcentration given earlier as Equation 3.123:

$$dC_f/dt = k_1C_w - k_2C_f \tag{3.140}$$

When expressed in fugacity terms, this same equation takes the following form:

$$VZD_f/dt = D_f f_w - D_f f_f \tag{3.141}$$

where V is the volume of the fish (m^3) and D_f is a fish-to-water transport parameter (mol/Pa/hr). Importantly, this equation implies that at steady state chemical fugacities in the water (f_w) and fish (f_f) are equal.

Extending this approach further, steady-state bioaccumulation in fish exposed to contaminated food and water may be calculated from the relationship:

$$D_f f_w + D_a f_a = f_f\left(D_w + D_e + D_m + D_g\right) \tag{3.142}$$

where $D_f f_w$ is uptake from water, $D_a f_a$ is uptake from food, and the transport parameters in the parentheses account for elimination into water (D_w; due to branchial efflux) and feces (D_e), biotransformation (D_m), and growth dilution (D_g). Using this approach, Campfens and Mackay (1997) concluded that fecal egestion and growth dilution are the major loss processes for high log K_{ow} compounds and that there is a net loss of chemical through the gills.

The principal advantage of a fugacity-based modeling approach is that thermodynamic relationships among compartments can be assessed using a common (fugacity) metric. When used for fish bioaccumulation modeling, the main disadvantage of this approach is that model parameters are not directly interpretable in terms of physiological quantities such as clearance and water and blood flows.

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4

Biotransformation in Fishes

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CONTENTS

Introduction

Biotransformation is a two-phase process catalyzed primarily through enzymatic reactions that often radically alter the chemistry of nonpolar lipophilic chemicals to polar water-soluble metabolites predominately leading to detoxification and elimination of the parent compounds. Unfortunately, the alteration of chemistry required for enhanced polarity often creates reactive intermediates through bioactivation, which can be more biologically hazardous than the initial parent compounds. The phase I process either adds or exposes polar atoms within a xeno- or endobiotic compound. Three general phase I reactions include oxidation, reduction, and hydrolysis (Table 4.1). When polarity has been enhanced through phase I reactions, phase II reactions generally attempt to further enhance polarity through conjugation of the phase I product with a bulky polar endogenous molecule. Alternatively, phase II reactions may protect against bioactivation by masking functional groups (i.e., amines) prone to reactive intermediate formation with groups that likely provide steric hindrance (i.e., methyl, acetyl) rather than augmented polarity (Table 4.1).

Phase I Reactions

Oxidation

Various enzymes are involved in the oxidation of xeno- and endobiotic compounds. Dehydrogenases oxidize substrates transferring electrons to an electron-deficient acceptor that is typically an essential cofactor for catalysis (e.g., NAD^+). Oxygenases catalyze the incorporation of molecular oxygen into molecules, and water is the source of oxygen for oxidases. Peroxidases derive oxygen from peroxide cofactors.

Cytochrome P450 Family of Drug Metabolizing Enzymes

Overview

The most dominant enzyme system responsible for oxidation processes in phase I biotransformation is the cytochrome P450 monooxygenases. The cytochrome P450s (CYPs) constitute a superfamily of heme-containing proteins that catalyze biological oxidation and reduction reactions. Klingenberg (1958) and Garfinkel (1958) first reported that hepatic microsomes contain a pigment that binds carbon monooxide with an unusual visible absorption maximum at 450 nm in its CO-reduced difference spectrum. Omura and Sato (1962) discovered that this pigment was a *b*-type cytochrome and called it cytochrome P450. The hepatic microsomal CYP system has broad substrate specificity and is responsible for oxidative metabolism of many structurally diverse endogenous and xenobiotic compounds. CYP enzymes are important for converting lipophilic foreign chemicals into more water-soluble products for excretion and, hence, detoxification. On the other hand, CYP enzymes catalyze the conversion of certain compounds such as polycyclic aromatic hydrocarbons (PAHs) and nitrosamines into more toxic intermediates. Constitutive CYP forms that appeared early in evolution are involved in biosynthesis (anabolism) of endogenous substances such as steroids, fatty acids, vitamins, bile acids, leukotrienes, thromboxanes,

TABLE 4.1

Phase I and Phase II Enzymatic Activities and Cofactors

Enzyme	Cofactors
Phase I	
Oxidation	
Cytochrome P450	Oxygen, NADPH, cytochrome b_5 (optional)
Flavin-containing monooxygenase	Oxygen, NADPH
Monoamine oxidase	H_2O
Aldehyde oxidase	NAD^+
Alcohol dehydrogenase	
Aldehyde dehydrogenase	
Cyclooxygenase	Arachidonic acid, oxygen
Peroxidase (PGH synthetase, lipoxygenase)	Peroxide (lipid-OOH or H_2O_2)
Reduction	
DT diaphorase	NAD(P)H
Hydrolysis	
Carboxylesterase	H_2O
Epoxide hydrolase	
Phase II	
UDP-glucuronosyl transferase	UDPGA
Sulfotransferase	PAPS
Amino acid conjugation	Amino acids (taurine, glycine, glutamine)
Glutathione *S*-transferase	Glutathione
Acetylation	Acetyl-coenzyme A
Methylation	*S*-Adenosylmethionine

and prostaglandins (Ryan and Levin, 1990). Inducible CYP forms (CYP1 through CYP4) emerged later in evolution and are primarily involved in the breakdown (catabolism) of endobiotics as well as xenobiotics. The following section attempts to update the phylogeny of each CYP family, addresses what is known regarding specific regulation, and then discusses what has been discovered regarding substrate specificities, catalytic function, and, when available, physiological role. Some families and isoforms have been better characterized than others. It is hoped that informational gaps are identified here that will stimulate further research in underrepresented CYP families and enhance our understanding of this important superfamily of enzymes.

The CYP superfamily is ancient, with the ancestral gene having existed more than 3.5 billion years ago (Nelson et al., 1993). Animals, plants, and microorganisms all contain CYP, and in mammals they have been identified in all tissues that have been examined. The emergence of new CYP genes results from a sequence of events, including speciation, gene duplication, divergence, and drift as a function of mutation and fixation all withstanding evolutionary pressures (Nebert et al., 1989; Nelsen, 1999). CYPs are generally most prevalent in the liver in association with the endoplasmic reticulum or mitochondria (Peter and Coon, 1991). As of 2004, the human genome has 57 putatively functional full-length *CYP* genes; *Fugu rubripes* (pufferfish) (Nelson, 2003) and *Danio rerio* (zebrafish) genomes have 54 and at least 81, respectively. CYP sequences that have been reported to the P450 nomenclature committee are listed on the cytochrome P450 homepage (http://drnelson.utmem.edu/CytochromeP450.html). The CYPs considered in the same family display more than 40% amino acid sequence similarity, and those within a subfamily are more than 55% similar (Nelson et al., 1993). Nomenclature has been standardized so *CYP* indicates the gene, followed by an Arabic numeral for the gene family, a capital letter for the subfamily, and an Arabic numeral for the specific subfamily member; for example *CYP*1A1 is responsible for the metabolic activation of benzo(*a*)pyrene (BaP) in most species, including mammals. The microsomal CYPs responsible for oxidation or metabolism of steroids and xenobiotic metabolism are located in families one through four.

The overall CYP-mediated reaction takes the form of:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

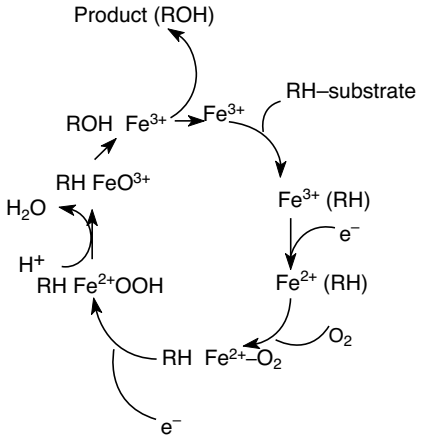
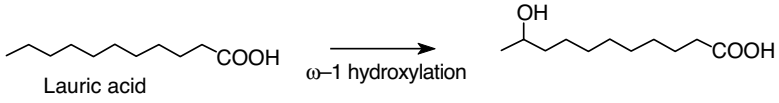
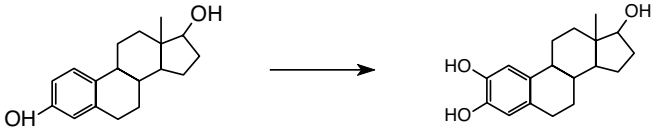


FIGURE 4.1 CYP catalytic cycle.

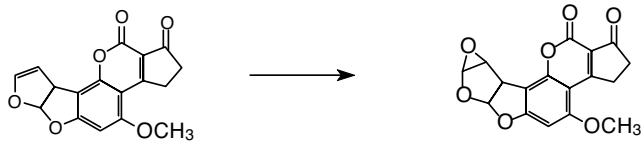
Hydroxylation of aliphatic carbons



Hydroxylation of aromatic carbons



Epoxidation



Heteroatom dealkylation

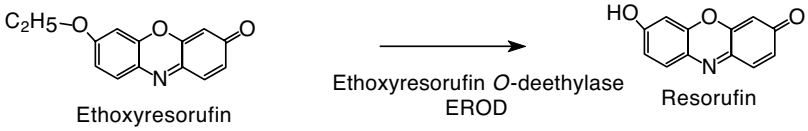


FIGURE 4.2A Examples of reactions catalyzed by cytochrome P450.

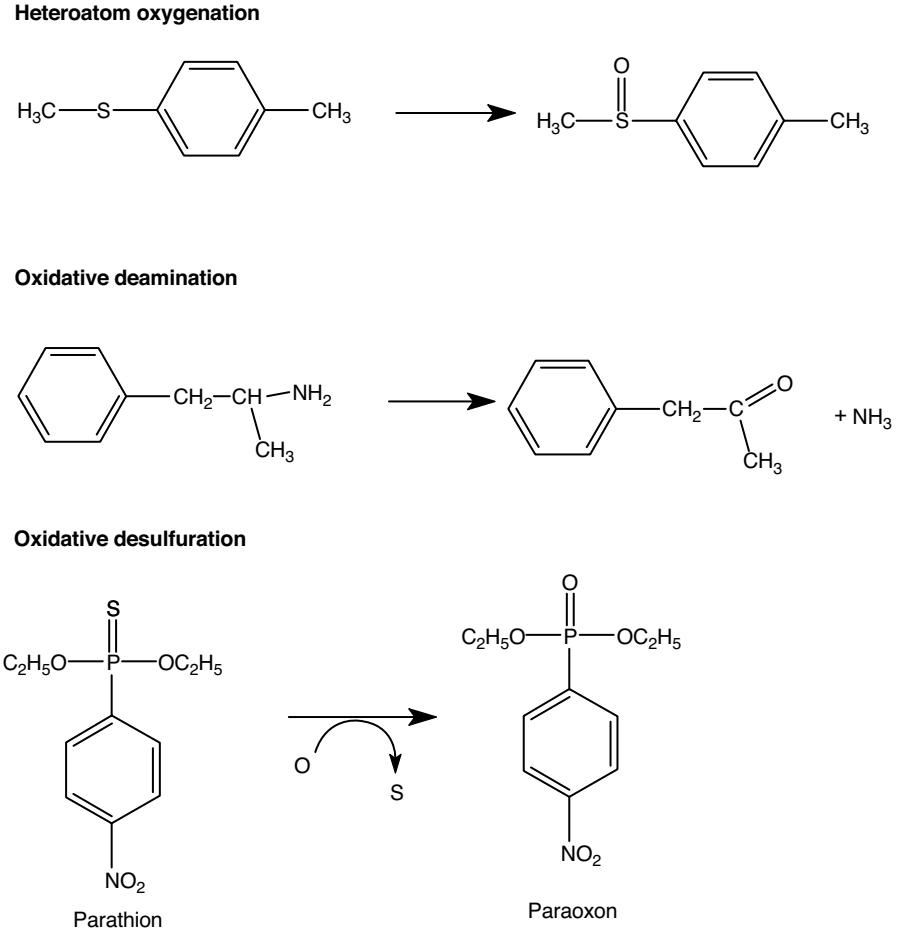


FIGURE 4.2B Examples of reactions catalyzed by cytochrome P450.

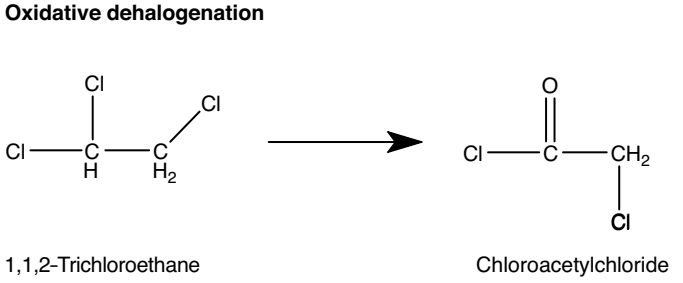


FIGURE 4.2C Examples of reactions catalyzed by cytochrome P450.

The reaction begins with the transfer of electrons from NAD(P)H to NADPH–cytochrome P450 reductase in the microsomal system (Figure 4.1). The active site of CYPs is generally a noncovalently bound heme iron in the form of protoporphyrin IX (Gonzalez and Gelboin, 1993; Henne et al., 1992). CYP-mediated reactions include aliphatic and aromatic carbon hydroxylation, epoxidation, heteroatom dealkylations, heteroatom oxygenation, deamination, desulfuration, and dehalogenation (Figure 4.2).

The reaction cycle is initiated when the substrate binds to the oxidized (Fe^{+3}) CYP complex and facilitates an electron transfer from NADPH to the complex. Oxygen then binds to the reduced CYP complex with coordination to iron *trans* to thiolate. The second electron is contributed by either

TABLE 4.2

CYP Gene Families, Inducers, Inhibitors, and Substrates

P450 Gene	Substrates	Inducers	Inhibitors
CYP1A	BaP Estradiol 7-Ethoxyresorufin Dimethyl benzanthracene Phenacetin	PAHs (BaP, BNF, 3-MC) Retene Dioxins/furans (TCDD) PCBs (CB77, 126, 169)	2-Aminoanthracene Elipticine PCB 77 Fluoranthene Cadmium Tributyltin α-Naphthoflavone Parathion Ketoconazole Miconazole Clotrimazole SKF525A
CYP1B	Estradiol (?)	BaP, TCDD	?
CYP1C	?	BaP, TCDD	?
CYP2K1	Lauric acid (ω–1) Aflatoxin B_1 17β-Estradiol Benzphetamine Progesterone (16α)	Diethyldithiocarbamate (activator) BNF (decrease) Testosterone (decrease mRNA) Estrogens (decrease protein)	Ketoconazole Miconazole Clotrimazole Cimetidine Parathion α-Naphthoflavone
CYP2M1	Lauric acid (ω–6) Progesterone	Estrogens (decrease protein)	?
CYP2N1	Arachidonic acid Benzphetamine Alkoxyresorufins	TPA/starvation (decrease mRNA)	?
CYP2N2	Arachidonic acid Benzphetamine Alkoxyresorufins	TPA/starvation (decrease mRNA)	?
CYP2P1	—	Fasting/refeed (increase mRNA)	?

NADPH–cytochrome P450 reductase or NADH–cytochrome b_5 reductase. The next step involves cleavage of the oxygen–oxygen bond, uptake of two protons, and release of water. Oxygen is inserted into the substrate through generation of hydroxyl and carbon free radicals. Finally, dissociation of ROH restores the P450 to the initial ferric state (Parkinson, 2001). In addition, the peroxide shunt can allow a peroxy compound to substitute for oxygen in substrate oxidation.

Expression of CYP genes are regulated by diverse mechanisms. Basal levels of individual CYP mRNAs and proteins are regulated via transcriptional and post-transcriptional processes, including mRNA and protein stabilization or degradation. Induction of CYP gene expression of isozymes in families one through three has been extensively investigated in vertebrates, including fish, and is described in the next sections of this chapter. Table 4.2 summarizes many inducers, inhibitors, and substrates of CYP1, CYP2, and CYP3 which can be used to measure induction of specific CYP isozyme-dependent activities in fish research. As shown in the table, many inducers, such as PAHs, are substrates for the CYPs that they induce and therefore stimulate their own metabolism. CYP-mediated metabolism of some substrates can be highly complex and is dependent on both the species and tissue or organ investigated. Figure 4.3 illustrates many of the oxidative metabolites of BaP that are catalyzed by CYP isoenzymes. The metabolite profiles are variable and dependent on species differences in expression of CYP isoforms and their catalytic activities; for example, fish (such as the brown bullhead) treated with BaP preferentially metabolize the hydrocarbon to the more toxic 7,8- and 9,10-oxidation products (Willett et al., 2000). In contrast, mussels exposed to BaP preferentially (47% total metabolites) form 1–6, 3–6, and 6–12 BaP quinones (Michel et al., 1995).

TABLE 4.2 (cont.)

CYP Gene Families, Inducers, Inhibitors, and Substrates

P450 Gene	Substrates	Inducers	Inhibitors
CYP2P3	Arachidonic acid Benzphetamine	TPA/starvation (decrease mRNA)	?
	Arachidonic acid Benzphetamine Alkoxyresorufins	TPA/starvation (decrease mRNA)	?
CYP2X1	Benzphetamine Aminopyrene	?	?
CYP3A27	Testosterone (6β,16β–OH) Progesterone Estradiol BaP Nifedipine Benzphetamine Ethoxycoumarin	Ketoconazole Estrogens (decrease)	α-Naphthoflavone Parathion Ketoconazole Miconazole Clotrimazole SKF525A Cimetidine Elipticine Piperonyl butoxide Isosafrole Gestoden 17α-Ethinylestradiol 1-Aminobenzotriazole 5,8,11,14-Eicosatetraynoic acid
CYP3A38	Testosterone (6β,16β–OH) Benzyloxyresorufin 7-Benzyloxy-4-[trifluoromethyl]-coumarin	Estrogens (decrease)	Nonylphenol (>100 n*M*)
CYP3A40	Testosterone (6β) Benzyloxyresorufin 7-Benzyloxy-4-[trifluoromethyl]-coumarin	—	—
CYP3A45	Testosterone (6β)	—	—

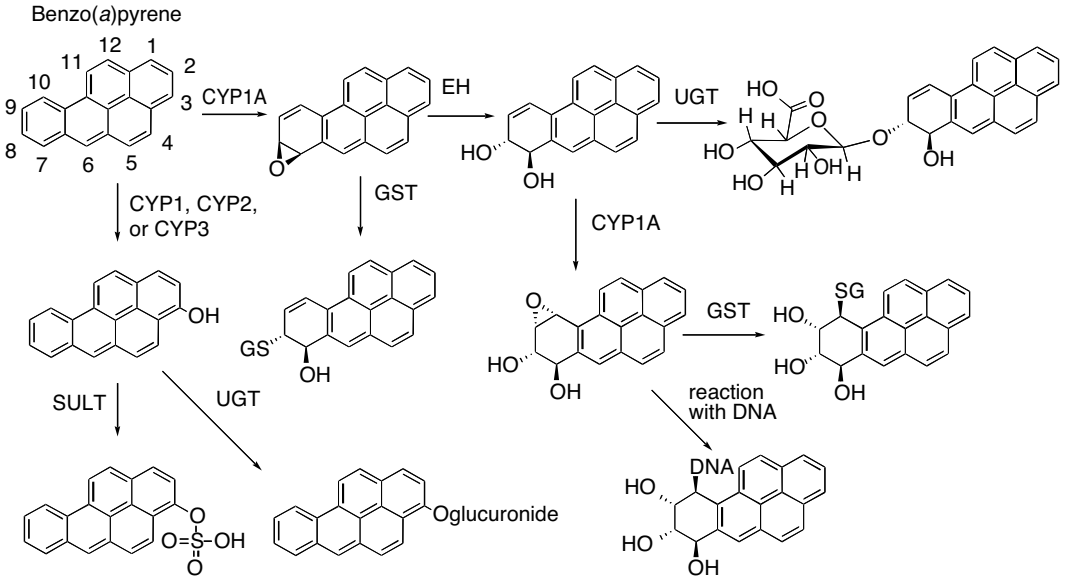


FIGURE 4.3 Biotransformation pathway of benzo(*a*)pyrene (BaP).

CYP1

Phylogeny — CYP1 split from the CYP2 family approximately 450 million years ago (MYA) (Nelson et al., 1993) (Figure 4.4). The CYP1 gene family represents one of the most studied families of CYP in fish, primarily with regard to the role that CYP1A subfamily members (see below) play in the biotransformation of environmentally persistent aromatic hydrocarbons (e.g., TCDD, PAHs) and their relationship with disease processes resulting from exposure to compounds of this nature (Stegman and Hahn, 1994).

Regulation—Expression of CYP genes are regulated by diverse mechanisms. Basal levels of individual CYP mRNAs and proteins are regulated via transcriptional and post-transcriptional processes, including mRNA and protein stabilization or degradation. CYP1 gene expression is induced by structurally diverse aromatic hydrocarbons wherein the induction response requires initial binding to the aryl hydrocarbon receptor (AhR). The AhR is a basic helix–loop–helix DNA-binding protein that has been extensively characterized in laboratory animals and human cell lines. In mammals, the AhR controls the transcription of the genes CYP1A1, CYP1A2, CYP1B1, as well as phase II enzymes such as glutathione *S*-transferase, uridine diphosphate (UDP)-glucuronosyltransferase, and aldehyde-3-dehydrogenase (Safe, 1995). Agonists for the AhR include both synthetic and naturally occurring compounds. The best characterized ligands include certain PAHs and planar halogenated aromatic hydrocarbons (HAHs), including polychlorinated dibenzo-*p*-dioxins (PCDDs) and -furans (PCDFs), as well as polychlorinated biphenyls (PCBs). Planar HAHs bind to the AhR in predictable structure–activity relationships. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has the highest AhR binding affinity among the HAHs and is also correlated with the high toxic potency of this congener (Safe, 1990). Structure–activity relationships are not as clear-cut for the PAHs because the toxic and genotoxic responses induced by these compounds are not all AhR mediated; however, Billiard and coworkers (2002) found a positive relationship between PAH binding to the teleost AhR and PAH potency for CYP1A induction. More recently, numerous naturally occurring dietary AhR ligands have been identified, including indole-3-carbinol, indolo-(3,2-*b*)-carbazole, dibenzoylmethanes, curcumin, and carotinoids (Denison and Nagy, 2003; Jeuken et al., 2003).

Studies with the $AhR^{-/-}$ knockout mouse have shown that this protein plays an important role in normal embryonic development (Abbott et al., 1995) and development of the liver and immune system (Fernandez-Salguero et al., 1995). The AhR was first identified in 1976 in hepatic cytosol from C57BL/6 mice (Poland et al., 1976). Apparent molecular masses of the photoaffinity-labeled cytosolic AhR are highly species dependent, ranging from 95 kDa for mouse to 124 kDa for hamster (Safe, 1995). The AhR has been identified by photoaffinity labeling in several species of teleosts and elasmobranchs (Hahn et al., 1994), as well as in the fish cell lines PLHC-1 and RTG-2 (Hahn et al., 1993). Nuclear AhR levels found in the killifish (*Fundulus heteroclitus*) were 203 fmol/mg, which was relatively high compared to most rodent species (Willett et al., 1995). Whereas mammals have a single AhR gene, two genes (AhR1 and AhR2) have been cloned in killifish (Karchner et al., 1999), zebrafish (Andreasen et al., 2002), and rainbow trout (*Oncorhynchus mykiss*) (Abnet et al., 1999). Four forms of the AhR (AhR1α, β; AhR2α, β) are found in Atlantic salmon (*Salmo salar* L.) (Hansson et al., 2004). The presence of AhR in fish indicates that this protein has been conserved for at least 450 million years. For more on AhR-mediated toxicities, refer to Chapter 5.

A simplified mechanism of AhR-mediated induction of CYP1A involves a ligand entering the cell where it associates with the cytosolic AhR, which exists as a multiprotein complex with two molecules of heat shock protein 90 (Hsp90), the X-associated protein 2 (XAP2), and a 23-kDa co-chaperone protein (p23) (Denison and Nagy, 2003). Binding of ligand causes a conformational change that facilitates nuclear translocation and association with the AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer, in turn, associates with dioxin response elements (DREs; sometimes called AhR response elements, or AhREs) and with various coregulators (Carlson and Perdew, 2002) to initiate transcription and translation of AhR-responsive genes, including CYP1s. Induction of CYP1 protein can be determined at the transcriptional (mRNA) level using northern blot or quantitative reverse transcription polymerase chain reaction (RT-PCR) and at the protein level by using western blot, enzyme-linked immunosorbent assays (ELISAs), or immunohistochemistry. Finally, CYP1-dependent catalytic activities such as aryl hydrocarbon hydroxylase (AHH) or ethoxyresorufin-*O*-deethylase (EROD) can also be used to measure induction.

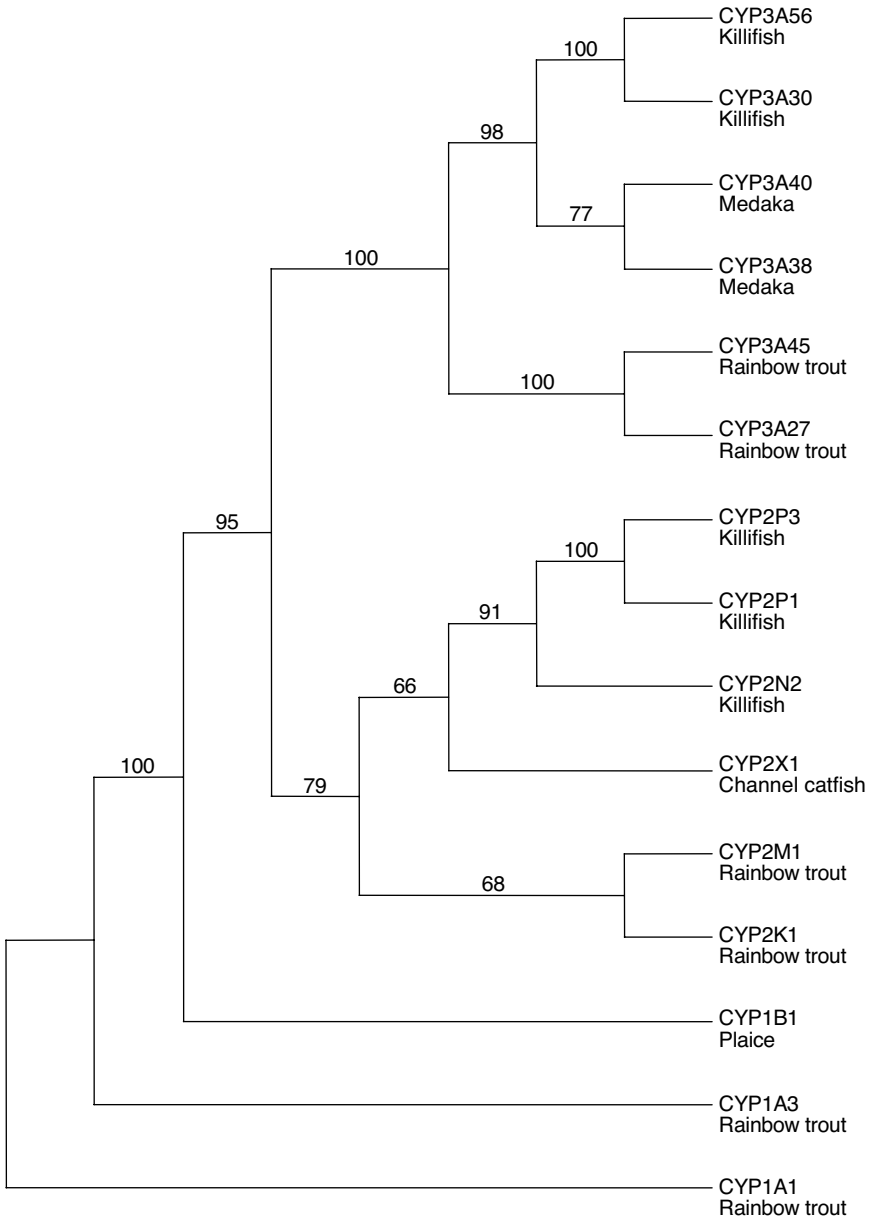


FIGURE 4.4 Phylogenic analyses of CYP in fish.

CYP1A—Research on CYPs in fish is more limited than that reported for mammals. Like mammals, fish CYPs catalyze oxidation of many of the same endogenous substrates and environmental chemicals. The most studied CYP form in fish is CYP1A. The first fish CYP1A cDNA was cloned by Heilmann et al. (1988), and sequence analysis showed 57 to 59% and 51 to 53% homology to mammalian CYP1A1 and CYP1A2 genes, respectively. This cDNA hybridized to a 2.8-kb mRNA that was induced 10-fold by 3-methylcholanthrene (3-MC) in rainbow trout. In fish, this form is generally recognized as CYP1A, and the terminology is supported because mammalian 1A1 and 1A2 are believed to have diverged 250 MYA by a gene-duplication event (Nebert and Gonzalez, 1987), whereas fish diverged from the mammalian line prior to that time. A hybrid CYP1A gene in fish is further suggested because some regions of the trout CYP1A sequence are identical to all mammalian CYP1A1 but not CYP1A2, while other

regions are identical to all mammalian CYP1A2 but not CYP1A1 (Stegeman and Hahn, 1994). This homology pattern can be explained by a single CYP1A gene in fish that is ancestral to both mammalian CYP1A1 and CYP1A2.

More recently, CYP1A has been cloned from killifish (Morrison et al., 1998) and medaka (*Oryzias latipes*) (Kim et al., 2004; Ryu et al., 2004) and assembled from the pufferfish (*Fugu rubripes*) genome, although the pufferfish sequence is missing much of its *N*-terminal half (Nelson, 1999). Morrison and coworkers (1998) conducted a phylogenetic analysis of CYP1 genes (mammalian, avian, and fish) that highlighted the problem with CYP1 nomenclature, particularly with trout CYP1A. Following the isolation by Heilmann et al. (1988) of the trout CYP1A1 cDNA, Berndtson and Chen (1993) reported a CYP1A2 gene cloned in rainbow trout; however, the sequence of this clone was only 4% different from the CYP1A1 clone isolated from the same species. Furthermore, the fish CYP1A2 was not orthologous to the mammalian 1A2 and was coordinately induced with CYP1A1 in trout treated with 3-MC (Berndtson and Chen, 1993). Later, the "CYP1A1" was renamed "CYP1A3," and the original "CYP1A2" was renamed "CYP1A1." Until functional data are provided for these various forms (Cao et al., 2000; Carvan et al., 1999), the nomenclature remains confusing and somewhat arbitrary, and, accordingly, CYP1As are not being provided a number following the subfamily until this issue is resolved (see cytochrome P450 homepage).

Multiple CYP proteins have been purified. Those corresponding to CYP1A include P450LM4b in rainbow trout (*Oncorhynchus mykiss*) (Williams and Buhler, 1984); P450E in scup (*Stenotomus chrysops*) (Park et al., 1986), perch (*Perca fluviatilis*) (Forlin and Celander, 1993), and rainbow trout (Celander and Forlin, 1991); and P450c in the Atlantic cod (*Gadus morhua*) (Goksøyr, 1985). The activity of the monoclonal antibody (mAb) developed against scup CYP1A (mAb 1-12-3) supports the conservation of CYP1A through evolution because this antibody recognizes presumptive CYP1A proteins in mammals, birds, amphibians, reptiles, and nearly 100 different fish species (Stegeman and Hahn, 1994). A study by Goksøyr and coworkers (1991) investigated the immunochemical cross-reactivity of the three antibodies prepared against the β-naphthoflavone (BNF)-inducible CYP1A proteins from rainbow trout, cod, and scup. Microsomes from induced hagfish (*Myxine* sp.), herring (*Clupea harengus*), rainbow trout, perch, scup, plaice (*Pleuronectes platessa*), and rat were tested. Western blot results indicate that all three antibodies recognize the same antigens in the microsomes, and, as expected, the antibodies react most strongly with their conspecific microsomes. Of the microsomes tested, perch and hagfish were the only microsomes not recognized by all three antibodies. The molecular mass of the immunoreactive proteins ranged from 52 kDa for hagfish to 59 kDa in rainbow trout (Goksøyr et al., 1991).

Since the early reports on the effects of crude oil on brown trout (*Salmo trutta*) (Payne and Penrose, 1975), research correlating environmental pollution with induction of CYP1A-mediated activities in fish has been used as a biomarker. The induction of hepatic CYP1A mRNA, immunoreactive protein, and EROD or AHH enzyme activities in fish have been extensively studied in both controlled laboratory and field experiments. Some of these studies are summarized in a review by Bucheli and Fent (1995), which found that 93% of the field studies (68/76) showed that CYP1A induction in fish was related to contaminant levels in the environment. It should also be noted that fish adapted to living in highly contaminated habitats (e.g., Superfund sites with PAH or HAH contamination) are also refractory to CYP1A induction (Bello et al., 2001; Brammell et al., 2004; Meyer et al., 2002; Prince and Cooper, 1995). Prolonged exposure of rainbow trout to a PCB mixture resulted in unresponsiveness to 3-MC treatment and decreased CYP1A expression upon additional dosage of PCB (Celander and Forlin, 1995). The mechanisms of this inhibition are not entirely clear (Meyer et al., 2003; Powell et al., 2000). For more on the use of CYP1A as a biomarker, refer to Chapter 16.

Inhibitors—The type, time dependence, and degree of inhibition caused by various CYP inhibitors are also species dependent. Selective inhibitors of CYP isozymes have been used to characterize substrate specificity and modulation of toxicity and carcinogenicity of xenobiotics and in biochemical mechanistic studies of CYP enzymes. Both AHH activities and the mutagenicity of 3-MC are inhibited by ellipticine and its derivatives. Structural analysis of ellipticine derivatives reveled that methyl substitution in the 5 and 11 positions is essential for inhibitory responses (Lesca et al., 1980). Some environmental contaminants are also CYP1A inhibitors in fish; for example, 2-aminoanthracene (2-AA) caused a 67% inhibition

of BNF-induced EROD activity in channel catfish (*Ictalurus punctatus*) (Watson et al., 1995). In both *in vitro* and *in vivo* experiments, 2-AA was a mechanism-based inhibitor of CYP1A. Similarly, 3,3′,4,4′-tetrachlorinated biphenyl (PCB 77) at high doses causes competitive inhibition of CYP1A enzyme activity and decreased induction of CYP1A protein (Gooch et al., 1989; White et al., 1997b). White and coworkers' (1997a) data suggested that, in scup, tetrachlorinated biphenyl (TCB) decreased CYP1A protein by enhancing protein degradation. TCB also initiated redox cycling through an uncoupling of CYP1A (Schlezinger and Stegeman, 2001). Finally, in killifish cotreated with BaP and the four-ring PAH fluoranthene (FL), both hepatic EROD activity and CYP1A immunoreactive protein levels were significantly inhibited (Willett et al., 2001). Although a covalent interaction between FL and CYP1A was not detected, the relative composition of DNA adducts changed in cotreated fish, suggesting that BaP metabolism is significantly affected when fish are co-exposed to PAH mixtures. Antifungal imidazoles (clotrimazole and ketoconazole) inhibit CYP1A-mediated EROD activities in gizzard shad (*Dorosoma cepedianum*), rainbow trout, and Atlantic cod (Hasselberg et al., 2004; Hegelund et al., 2004; Levine et al., 1997). Acrylamide, an environmental contaminant, is also an inhibitor of CYP1A and suggests feedback regulation on CYP1A mRNA transcription (Haasch et al., 1992; Petersen and Lech, 1987).

CYP1B—Until the mid-1990s, the CYP1 gene family was believed to contain a single subfamily with the well-known members CYP1A1 and CYP1A2; however, in 1994, the cDNA for CYP1B1 was isolated from TCDD-induced human keratinocyte cells (Sutter et al., 1994). Human CYP1B1 is a single-copy gene that is located on chromosome 2. It contains three exons and two introns and generates a 5.2-kb mRNA (Murray et al., 2001). In mammals, CYP1B1 expression is high in vascular endothelial cells, breast, prostate, uterus, epithelial lining of the head and neck, and the adrenal cortex (Nebert et al., 2004). CYP1B1 protein has also been reported in human breast, kidney, lung, brain, and testis tumors (McFadyen et al., 2001; Murray et al., 2001). Recombinant human CYP1B1 is highly active in oxidizing the potent PAHs BaP and dimethylbenzanthracene (DMBA) to their respective carcinogenic metabolites. Shimada and coworkers (1999) found that CYP1B1 was more active than CYP1A1 in metabolizing BaP to the proximate toxicant BaP-7,8-diol. Their study suggests that species or tissues with less CYP1B1 may be less likely to form DNA-reactive PAH metabolites and thereby may be more resistant to carcinogenesis. This finding is supported by studies with CYP1B1-null mice. Seventy percent of DMBA-treated wild-type mice developed highly malignant lymphomas, whereas the CYP1B1-null mice only had 7.5% cancer incidence (Buters et al., 1999). Likewise, metabolism of DMBA to toxic intermediates in MCF7 and T47D breast cancer cells is blocked by CYP1B1 antibodies (Angus et al., 1999; Christou et al., 1994).

A significant void in CYP1B1 research exists in nonmammalian species such as fish. There are only two published studies where CYP1B was studied in four fish species: scup, killifish, zebrafish (*Danio rerio*) (Godard et al., 2000), and plaice (Leaver and George, 2000). The plaice CYP1B has two protein-coding exons with similar exon–intron boundaries compared to the human CYP1B1. The amino acid sequence of the plaice has 54% identity with the human CYP1B1 sequence but only 39% identity with the plaice CYP1A (Leaver and George, 2000). CYP1B was detected in plaice gill by northern blot but did not appear to be induced by BNF. For carp (*Cyprinus carpio*), two partial CYP1B and two CYP1C sequences have been submitted to GenBank. Similarly, scup genes and the killifish partial sequence recently have been reclassified CYP1C1 and CYP1C2, suggesting that the genes have less than 55% amino acid identity with either the plaice or mammalian CYP1Bs. The cloned channel catfish and brown bullhead (*Ameiurus nebulosus*) CYP1B genes are 71, 61, and 55% similar, respectively, to carp, plaice, and human CYP1B1 (accession number DQ088663). Currently, none of the CYP1B-dependent metabolic studies has been done in fish or fish cells, yet fish are commonly used in toxicology and carcinogenicity testing. *In vivo* and *in vitro* data in catfish do indicate that CYP1B mRNA is induced in gill (but not liver) primary cultured cells and gill, kidney, blood, gonad, and liver following BaP exposure (Butala, unpublished data; Metzger, unpublished data).

In addition to its involvement in PAH metabolism, human CYP1B1 is an estradiol hydroxylase primarily at the C-4 position, whereas CYP1A1 and CYP1A2 have activity at the C-2, C-6α, and C-15α positions of estradiol (Hayes et al., 1996). Tumors have been reported in tissues where estradiol was converted to the 4-hydroxyestradiol metabolite, but tumors did not form where there was primarily

2-hydroxylation. Similarly, an elevated ratio of 4-/2-hydroxyestradiol formation in human mammary microsomes has been used as a risk factor for malignant breast cancer (Liehr and Ricci, 1996). Catechol estrogens, such as 4-hydroxyestradiol, are capable of undergoing metabolic redox cycling between hydroquinone and quinone forms, generating potentially mutagenic free radicals and oxidative stress. In channel catfish the 4-/2-hydroxyestradiol ratio was statistically higher in microsomes from BaP-treated fish compared to controls (0.2 and 0.04, respectively) (Butala et al., 2004). The shift toward more 4-hydroxyestradiol production in exposed fish suggests induced production of the redox active estrogen metabolite. As with PAH metabolism, lower levels of CYP1B1 and less associated formation of 4-hydroxyestradiol may be indicative of lower genotoxicity. More studies are necessary to determine the physiological significance of CYP1B in fish.

CYP2

Phylogeny — CYP2 and CYP1 genes belong to the CYP2 clan (Figure 4.4). The CYP1 gene family is believed to have diverged from the CYP2 gene family more than 420 MYA (Nelson, 2003). CYP2 is the most diverse CYP gene family, with 13 known CYP2 subfamilies in fish: 2K, 2M, 2N, 2P, 2R, 2U, 2V, 2X, 2Y, 2Z, 2AA, 2AD, and 2AE (see cytochrome P450 homepage). Two of these, CYP2R and CYP2U, also have mammalian representatives; thus, these subfamilies probably represent more conserved CYP2 genes that emerged over 420 million years of evolution. No functional data are available on CYP2R and CYP2U isozymes, although it is proposed that these earlier emerging CYP2 forms are more likely involved in the metabolism of endobiotics than xenobiotics (Nelson, 2003).

Although mammalian and piscine CYP2 gene families have structurally diverged during vertebrate evolution, there are still some conserved structures as well as catalytic functions among certain CYP2 subfamilies; for example, fish CYP2N, CYP2P, CYP2V, and CYP2Z genes are related to mammalian CYP2D and CYP2J genes (Nelson, 2003). Furthermore, phylogenetic analyses suggest that the killifish CYP2P subfamily is more closely related to the mammalian CYP2J subfamily, compared to the CYP2K and CYP2N subfamilies in fish; thus, the piscine CYP2P and the mammalian CYP2J subfamilies may have arisen from a common ancestral gene (Oleksiak et al., 2003). The fish CYP2 genes and suggested pseudogenes reported to the P450 nomenclature committee are listed in Table 4.3.

Members of the CYP2 gene family in fish are involved in the metabolism of endobiotics, such as arachidonic acid, lauric acid, and sex steroid hormones, as well as xenobiotics such as aflatoxin, alkoxyresorufins, and benzphetamine (Buhler and Wang-Buhler, 1998; Oleksiak et al., 2003; Yang et al., 1998, 2000). The following paragraphs summarize highlights from functional studies of some of these CYP2 subfamilies.

CYP2B-Like Forms—Over the last decades, fish liver microsomes have been shown to metabolize prototypical mammalian CYP2B substrates, including aldrin, benzphetamine, ethylmorphine, aminopyrine, and alkoxyresorufins (Buhler and Williams, 1989; Eisele et al., 1984; Elskus and Stegeman, 1989; Goksøyr et al., 1987; Haasch et al., 1994; Kleinow et al., 1990; Stegeman, 1981). The existence of piscine CYP2B-like enzymes further was supported by protein purification and immuno-cross-reactivity studies; however, a piscine CYP2B gene ortholog has so far not been reported. In the scup, five different CYP isozymes (P450A to P450E) were isolated from liver microsomes. Reconstitution of scup P450B demonstrated oxidation of testosterone at the 15α-position (Klotz et al., 1986). *N*-terminal analysis of scup P450B showed 50% sequence identity with rat CYP2B1 and CYP2B2; furthermore, proteins from different taxa, including several fish species, show cross-reactivity with antibodies against both scup P450B and rat CYP2B1 (Stegeman and Hahn, 1994).

In rainbow trout, five different CYP isozymes (LMC1 to LMC5) were isolated from liver microsomes (Miranda et al., 1989). Reconstituted LMC1 was shown to catalyze lauric acid hydroxylase activity and to cross-react with rat CYP2B1 antibodies (Miranda et al., 1989, 1990). Rainbow trout LMC1 was later assigned as CYP2M1 (Yang et al., 1998). Further studies are needed to elucidate whether scup P450B and CYP2M1 are homolog genes and whether the mammalian CYP2B and the piscine CYP2M subfamilies have arisen from a common ancestral gene.

The presence of hepatic CYP2B immunoreactive proteins and comparatively high activities toward mammalian prototypical CYP2B substrates (i.e., aminopyrene and pentoxyresorufin) were observed in

TABLE 4.3

CYP2 Gene Family in Fish

Subfamily/Gene	Species	Refs.
CYP2K	Rainbow trout (*O. mykiss*)	Buhler et al. (2000); Cok et al. (1998);
2K1	Killifish (*F. heteroclitus*)	Katchamart et al. (2002); Yang et al. (2000)
2K2	Rainbow trout	
2K3-2K5	Zebrafish (*D. rerio*)	
2K6-2K8; 2K16-2K22	Pufferfish (*F. rubripes*)	
2K9-2K11	Pufferfish	
2K12-2K15 pseudogenes		
CYP2M	Rainbow trout	Buhler et al. (2000); Cok et al. (1998);
2M1		Katchamart et al. (2002); Yang et al. (1998)
CYP2N	Killifish	Oleksiak et al. (2000); Peterson and Bain (2004);
2N1	Killifish	Schlenk et al. (in press); Vrolijk et al. (1994)
2N2	Scup (*S. chrysops*)	
2N3	Butterfly fish (*Chaetodon* sp.)	
2N4-2N8	Pufferfish	
2N9-2N11	Zebrafish	
2N13		
CYP2P	Killifish	Oleksiak et al. (2003)
2P1-2P3	Pufferfish	
2P4	Pufferfish	
2P5 pseudogene	Zebrafish	
2P6-2P10	Largemouth bass (*M. salmoides*)	
2P11	Atlantic salmon (*S. salar*)	
2P		
CYP2R	Zebrafish	
2R1	Pufferfish	
2R1	Pufferfish	
2R2-2R3 pseudogenes		
CYP2U	Zebrafish	
2U1	Pufferfish	
2U1		
CYP2V	Zebrafish	
CYP2V1		
CYP2X1	Catfish (*I. punctatus*)	Schlenk et al. (2002)
CYP2X1	Pufferfish	
2X2-2X4	Pufferfish	
2X5 pseudogene	Zebrafish	
2X6-2X11		
CYPY	Pufferfish	
2Y1-2Y2	Zebrafish	
2Y3-2Y4		
CYPZ	Pufferfish	
2ZI-2Z2		
CYP2AA		
2AA1-2AA8		
CYP2AD	Pufferfish	
2AD1 (formerly 2N12)	Zebrafish	
2AD2-2AD3; 2AD6	Medaka (*O. latipes*)	
2AD4	Three-spined stickleback	
2AD5	(*Gasterosteus aculeatus*)	
CYP2AE	Zebrafish	
2AE1		

Note: Genes, gene fragments, and possible pseudogenes were obtained from the cytochrome P450 homepage: http://drnelson.utmem.edu/CytochromeP450.html.

some tropical fishes from the Bermuda Archipelago; however, great differences were found in aminopyrene-*N*-demethylase and pentoxyresorufin-*O*-depentylase activities among species, with the Bermuda chub (*Kyphosous sectatrix*) and sergeant major (*Abudefduf saxatilis*) displaying the highest activities. In addition, polyclonal antibody (pAb) against rat CYP2B1 cross-reacted most strongly with hepatic microsomal proteins in tomtate (*Haemulon aurolineatum*), pinfish (*Lagodan rhomboides*), Bermuda chub, and sergeant major (Stegeman et al., 1997). The reason for the observed differences in CYP2B-like expression among species is not known. Earlier, it was suggested that natural dietary compounds may be causing these differences, as higher CYP2B-like protein levels were observed in butterfly fish (*Chaetodon capistratus*) that consumed gorgonians (containing high levels of allelochemicals) compared to butterfly fish that avoided gorgonians (Vrolijk et al., 1994). CYP2N mRNA expression in *Chaetodon xanthurus* (CYP2N7) was significantly higher than in the facultative coralline-feeding butterfly fish *C. kleini*, *C. auriga* (CYP2N6), or *C. vagabundus*, as well as an obligate coralline feeding species (*C. punctofasciatus*) from the Great Barrier Reef in Australia (DeBusk, 2001). When each species, including *C. xanthurus*, was gavaged with gorgonian extracts from *Sinnularia maxima* for 3 days, with the exception of *C. punctofasciatus*, CYP2N mRNA expression was diminished (DeBusk, 2001). In the Bermuda species investigated, herbivorous fish had higher CYP levels (including CYP2B-like proteins) compared to carnivorous fish (Stegeman et al., 1997). It remains to be shown if natural dietary chemicals may act as inducers of CYP2B-like forms in fish or if other mechanisms are involved.

Phenobarbital (PB) and 1,4-*bis*(2-[3,5-dichloropyridyloxy])benzene (TCPOBOP) are powerful PB-type inducers of CYP2B genes in mammals (Poland et al., 1981). In mammals, induction of CYP2B by PB-type inducers proceeds through activation of the constitutive androstane receptor (CAR) followed by nuclear translocation, dimerization with the retinoid X receptor (RXR), and binding to phenobarbital response elements (PBREMs) in the promoter region of the CYP2B genes (Honkakoski et al., 1997, 1998a,b).

In fish, however, an apparent lack of response to PB-type inducers has been observed (Buhler and Williams, 1989; Eisele et al., 1984; Goksøyr et al., 1987; Haasch et al., 1994; Iwata et al., 2002; Stegeman, 1981), although a CAR immunoreactive protein was detected in scup liver cytosol and nucleus using antibodies against human CAR. No induction of CYP protein levels, including scup P450B, or catalytic activities were seen in scup injected with TCPOBOB. In fact, TCPOBOB treatment had no effect on translocation of the cytosolic CAR-immunoreactive protein in scup liver (Iwata et al., 2002). This study points to functional differences, possibly in receptor activation or translocation, between fish and mammals. Recently, a single piscine CAR/PXR gene was identified (*fr078207*) when searching the pufferfish genome; however, this receptor was more related to PXR family members and hence a probable functional analog of PXR (Maglich et al., 2003). Thus, CAR may have diverged from the pregnane X receptor (PXR) at a later point in vertebrate evolution, or CAR may have been lost in some or all teleost lines (Maglich et al., 2003). The apparent lack of a piscine CAR receptor may be one explanation for the observed lack of PB-type as well as diminished CYP3A (see below) induction in fish.

CYP2E-Like Forms—The possible existence of a CYP2E form in fish (*Poeciliopsis monacha-lucida*) was proposed based on hybridization with a rat CYP2E1 49-base oligonucleotide, antibodies to rat CYP2E1, as well as responsiveness to ethanol treatment. This CYP form was suggested to be involved in the CYP-mediated dealkylation of the fish carcinogen diethylnitrosamine (Kaplan et al., 1991). Furthermore, hepatic microsomal metabolism of the mammalian CYP2E substrate chlorzoxazone in winter flounder (*Pleuronectes americanus*) and in viviparous *Poeciliopsis monacha* and *Poeciliopsis viriosa* is indicative of the presence of CYP2E-like enzymes (Kaplan et al., 2001; Wall and Crivello, 1998). A piscine CYP2E gene ortholog, however, has so far not been reported.

The CYP2K Subfamily—A CYP protein, denoted LMC2, was isolated from rainbow trout liver (Miranda et al., 1989). It was subsequently cloned and assigned as CYP2K1 (Buhler et al., 1994). CYP2K1 is one of the dominant CYP forms expressed in liver and trunk kidney, and it displays sexually dimorphic expression, with higher levels in sexually mature males compared to females (Buhler et al., 1994). In addition to liver and trunk kidney, CYP2K1 also is expressed, though at lower levels, in blood cells, upper intestine, head kidney, stomach, heart, gonads, and male muscle (Cok et al., 1998). Heterologous

expression of CYP2K1, using baculovirus *Spodoptera frugiperda* (*Sf9*)-infected insect cells, showed that CYP2K1 catalyzed hydroxylation of lauric acid primarily at the (ω-1) position but also to a minor extent at the (ω-2) position. In addition, CYP2K1 catalyzes the conversion of the procarcinogen aflatoxin B_1 to aflatoxin B_1-8,9-epoxide (Yang et al., 2000). Treatment of rainbow trout with 17β-estradiol decreased CYP2K1 mRNA and protein levels as well as lauric acid hydroxylase activities and bioactivation of aflatoxin B_1. Treatment with testosterone resulted in slightly decreased CYP2K1 mRNA levels, whereas this treatment had no significant effect on CYP2K1 protein levels (Buhler et al., 2000). Thus, CYP2K1 is mainly expressed in the digestive tract and appears to be involved in metabolism of endobiotics such as lauric acid as well as xenobiotics such as aflatoxin B_1. In addition, CYP2K1 also is expressed in, for example, steroidogenic tissues; furthermore, hepatic CYP2K1 expression is affected by sex steroids, particularly 17β-estradiol (Buhler et al., 2000; Cok et al., 1998). This may have toxicological implications, as many natural fish populations are exposed to endocrine-disrupting chemicals (EDCs), including estrogenic compounds. In fact, treatment of rainbow trout with xenoestrogens (methoxychlor, diethylstilbestrol, 4-*tert*-octylphenol, and biochanin A) decreased hepatic acid hydroxylase activity and reduced CYP2K1 protein expression (Katchamart et al., 2002). CYP2K gene orthologs also have been cloned from killifish, pufferfish, and zebrafish (see Table 4.2 and Table 4.3).

The CYP2M Subfamily—As mentioned earlier, rainbow trout LMC1, previously isolated from rainbow trout liver (Miranda et al., 1989), was cloned and assigned as CYP2M1 (Yang et al., 1998). Highest expression of CYP2M1 was observed in liver, but also trunk kidney expresses CYP2M1. Interestingly, in trunk kidney a pronounced sexually dimorphic expression of CYP2M1 was seen, with juvenile females expressing 20-fold higher levels than juvenile males. Expression decreased in trunk kidney in sexually mature animals (Yang et al., 1998). In addition to liver and trunk kidney, CYP2M1 also is expressed, though at lower levels, in head kidney, stomach, heart, gonads, and brain (Cok et al., 1998). Recombinant CYP2M1, expressed in *p*SLV-transfected COS-7 and in baculovirus-infected *Sf9* cells, catalyzed hydroxylation of lauric acid primarily at the (ω-6) position (Yang et al., 1998). Although CYP2M1 appears to be involved in the metabolism of fatty acids and fatty acid derivatives, the physiological function is unknown. Treatment of rainbow trout with 17β-estradiol resulted in decreased CYP2M1 protein and mRNA levels, whereas treatment with testosterone had no effect on CYP2M1 (Buhler et al., 2000). In addition, treatment with xenoestrogens (methoxychlor, diethylstilbestrol, 4-*tert*-octylphenol, and biochanin A) also reduced CYP2M1 protein levels as well as lauric acid hydroxylase activity (Katchamart et al., 2002). It should be noted, however, that the lauric acid hydroxylase activity assay used could not distinguish between CYP2K1, CYP2M1, and possible other CYP activities. So far, no CYP2M ortholog genes have been reported in other species (Table 4.3); however, increased renal ω and ω-6 hydroxylation of lauric acid was observed in male channel catfish treated with ciprofibrate, whereas in male bluegill (*Lepomis macrochirus*) hepatic ω, ω-4, and ω-5 hydroxylation was induced by ciprofibrate (Haasch et al., 1998). Although sequence data are needed to verify the identity of the CYP enzymes induced, these data imply induction of CYP2M-like activities by peroxisome proliferators.

The CYP2N Subfamily—CYP2N1 and CYP2N2 were cloned from killifish liver and heart cDNA libraries (Oleksiak et al., 2000). CYP2N1 mRNA expression was detected at high levels in liver and intestine (mid gut) and at low levels in heart and brain. CYP2N2 mRNA levels were highest in heart and brain and present at lower levels in liver and intestine. Heterologous expression of CYP2N1 and CYP2N2 in *Sf9* insect cells revealed transformation of arachidonic acid to epoxyeicosatrienoic acids. CYP2N1 preferentially metabolized arachidonic acid at the 8,9- and 11,12-olefins and to a lesser degree at the 14,15-olefin, and CYP2N2 preferentially metabolized arachidonic acid at the 8,9-olefin and to a lesser degree at the 11,12- and 14,15-olefins. In addition to being arachidonic acid epoxygenases and hydrolases, the CYP2Ns also metabolize xenobiotics. Thus, both CYP2N1 and CYP2N2 isoforms metabolize benzphetamine and also exhibit minimal alkoxyresorufin-*O*-dealkylase activities (Oleksiak et al., 2000). Intestinal CYP2N1 mRNA levels were decreased in starved animals and in animals treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or starved and treated with TPA-treated killifish. Intestinal CYP2N2 mRNA levels were diminished in animals treated with TPA, whereas starvation had no significant effect on this response; however, increased CYP2N2 mRNA expression was observed in

heart (but not in brain) in killifish treated with TPA (Oleksiak et al., 2000). Hepatic CYP2N2 mRNA levels were increased in killifish exposed to anthracene in the lab and in killifish collected from a PAH-contaminated site (Peterson and Bain, 2004); thus, CYP2N genes are expressed in hepatic and extrahepatic organs and appear to be regulated by environmental agents. CYP2N gene orthologs also have been cloned from scup, butterfly fish (*Chaetodon xanthurus*), pufferfish, and zebrafish (Table 4.3).

The CYP2P Subfamily—CYP2P1, CYP2P2, and CYP2P3 were cloned from a killifish genomic library (Oleksiak et al., 2003). CYP2P genes are predominantly expressed in liver and intestine. Recombinant CYP2P3 expressed in baculovirus-infected *Sf9* insect cells catalyzed benzphetamine *N*-dealkylation and arachidonic acid oxidation. Arachidonic acid was oxidized to 14,15-, 11,12-, and 8,9-epoxyeicosatrienoic acids and 19-hydroxyeicosatetraenoic acid. The regiospecificity was similar to human CYP2J2 and rat CYP2J3 isozymes. Similar to that observed for CYP2N1 and CYP2N2, decreased levels of intestinal CYP2P2 and CYP2P3 mRNA were observed in killifish treated with TPA. Prolonged starvation or fasting (20 days) resulted in decreased levels of CYP2P2 and CYP2P3. Neither TPA nor fasting had any significant impact on CYP2P1 mRNA levels; however, fasting followed by refeeding resulted in increased levels of CYP2P1 mRNA but not CYP2P2 and CYP2P3 transcription levels. In rat, fasting resulted in reduced intestinal CYP2J3 and CYP2J4 protein levels; thus, intestinal expression of killifish CYP2P2 and CYP2P3 genes and arachidonic acid regiospecificity of the recombinant CYP2P3 isozyme show great similarities to mammalian CYP2J forms. Relatedness to mammalian CYP2J genes was further confirmed by phylogenetic analysis, using the minimum evolution criterion. Killifish CYP2P genes clustered with mammalian CYP2J genes, separate from the piscine CYP2N and CYP2K subfamilies (Oleksiak et al., 2003). CYP2P gene orthologs also have been cloned from scup, butterfly fish, pufferfish, zebrafish, and largemouth bass (*Micropterus salmoides*) and as a partial sequence from Atlantic salmon (Table 4.3).

The CYP2X Subfamily—The presence of two CYP2-like isozymes in channel catfish was indicated based on immunoreactivity with antibodies against both rainbow trout CYP2K1 and CYP2M1. Furthermore, treatment with ethanol (Perkins and Schlenk, 1998) or the insecticide fenitrothion (Perkins, 1999) specifically decreased expression of the lower (47-kDa) protein. Clofibrate treatment specifically increased the upper (51-kDa) protein. Furthermore, female catfish displayed higher levels of the 47-kDa protein compared to males (Perkins, 1998). A CYP2-immunoreactive protein, denoted CM-HA3, was next isolated from channel catfish liver. *N*-Terminal amino acid analyses followed by a BLAST search revealed sequence identity to both CYP2K1 and CYP2M1 (Perkins et al., 2000). By using degenerate PCR primers designed against this *N*-terminal followed by RACE, a CYP clone was isolated from catfish liver and designated as CYP2X1 (Schlenk et al., 2002); however, the derived amino acid sequence was different from the *N*-terminus of CM-HA3. CYP2X1 expressed in *Sf9* cells demonstrated benzphetamine demethylase activity, but testosterone, fenthion, and *p*-nitrophenol metabolism was not observed. CYP2X gene orthologs also have been cloned from pufferfish and zebrafish (Table 4.3).

CYP3

Phylogeny — Analyses of the complete sequences of several teleost genomes indicate that fish species contain a complement of CYP gene families similar to those found in mammals. To date, however, tissue distribution, mechanisms of gene regulation, and the catalytic function of many of these enzymes remain unknown. The CYP3 gene family is believed to have diverged between 800 and 110 MYA (Maurel, 1996), and four subfamilies have been identified, including CYP3A to CYP3D (see CYP home page). To date, 13 teleost CYP3A genes have been identified by sequence homologies. Additional subfamilies (CYP3B to CYP3D) have been discovered by data mining of both the pufferfish and zebrafish genome databases (see Table 4.4). The CYP3 identity of these genes was based on gene sequence homologies of less than 55% when compared to members of the CYP3A family. Current nomenclature for the CYP3 gene family, however, does not reflect orthologous relationships between organisms due to the presence of multiple CYP3A-like sequences in individual species; thus, CYP3A diversity is thought to include both orthology (diversification due to speciation) and paralogy (diversification due to gene duplication) (McArthur et al., 2003; Nelson et al., 1996). In a previous study, orthologous relationships between

mammalian and teleost CYP3A genes were suggested using nearest-neighbor and maximum parsimony methods (Celander and Stegeman, 1997). More recently, Bayesian analysis of 45 vertebrate CYP3A deduced amino acid sequences suggest that teleost, diapsid, and mammalian CYP3A genes have undergone independent diversification and that an ancestral vertebrate genome contained a single CYP3A gene (Hegelund and Celander, 2003; McArthur et al., 2003). Phylogenetic analyses suggest that the divergence of CYP3A paralogs and additional subfamily members is likely due to successive gene-duplication events. Whole genome duplications in teleosts have been suggested (Christoffels et al., 2004; Furutani-Seiki and Wittbrodt, 2004), and multiple CYP3A paralogs have been identified in several species, including medaka, rainbow trout, and killifish (Celander and Stegeman, 1997; Hegelund and Celander, 2003; Kullman and Hinton, 2001; Kullman et al., 2000; Lee and Buhler, 2003; Lee et al., 1998; Lemaire et al., 1996). Teleost CYP3A paralogs demonstrate high degrees of sequence similarity: 90% (CYP3A38 and CYP3A40), 94% (CYP3A27 and CYP3A45), and 98% (CYP3A30 and CYP3A56) for medaka, trout, and killifish, respectively. Each sequence conforms to the specific structural features associated with the cytochrome CYP gene superfamily and exhibits >40% sequence similarity to the CYP3A subfamily. It has been suggested that the topologies of all CYP enzymes are similar, especially regarding structurally conserved regions such as the heme-binding domain, oxygen-binding region, and specific sites associated with redox interactions (Szklarz and Halpert, 1997). Differences in CYP catalytic activities are suggested to be determined predominantly by amino acid composition in six substrate recognition sites (SRS1 to SRS6) (Gotoh, 1992). Recently homology models for CYP3A genes have been described (Harlow and Halpert, 1998; Yang et al., 1998). Key amino acids associated with CYP3A substrate specificity, binding, and regio-specific catalysis have been suggested by using molecular modeling and site-directed mutagenesis. Statistical comparisons using the DIVERGE program identified regions in SRS1, SRS5, and SRS6 that appear to be associated with a general conserved CYP3A function, whereas SRS2, SRS3, and SRS4 confer functional differences among different CYP3A enzymes (McArthur et al., 2003). Alignments of medaka CYP3A38 and CYP3A40 demonstrate that 12 of 49 amino acid differences occur in SRS regions. These differences are predominately observed in SRS1, SRS3, and SRS6. As noted below, it has been suggested that these amino acid substitutions are responsible for the differing kinetic and catalytic properties of these two teleost paralogs.

Multiple CYP3A-like teleost proteins have additionally been observed using immunochemical detection in numerous other species. Although gene sequences for these species have not been identified, cross-reactivity with antibodies specific for either mammalian or teleost CYP3A proteins suggests that multiple CYP3A-like proteins are present in the liver and intestine of several teleosts (Celander et al., 1996).

Function—Functionally, CYP3A enzymes are among the most versatile forms of CYPs as they have unusually broad substrate specificities for both endogenous and exogenous substrates, including steroids, bile acids, eicosanoids, retinoids, xenobiotics such as pharmaceuticals, and procarcinogens (Aoyama et al., 1990; Gillam et al., 1993; Li et al., 1995; Smith et al., 1996; Waxman et al., 1998). CYP3A-like proteins were initially purified from several teleost species, including scup, rainbow trout, and Atlantic cod (Celander et al., 1989; Klotz et al., 1986; Miranda et al., 1989). Identification of these proteins as CYP3A-like was based predominantly on steroid hydroxylase activity and cross-reactivity with CYP3A-specific antibodies. In some instances, antibodies were additionally used as catalytic inhibitors. Purified cytochrome P450A from scup and LMC5 from rainbow trout exhibited specific steroid hydroxylase activity, similar to that observed with mammalian CYP3A enzymes. Each enzyme additionally demonstrated minimal benzo(*a*)pyrene hydroxylase and ethoxycoumarin *O*-deethylase activities, suggesting a functional difference from the previously identified and inducible CYP1A form. Further characterization of purified teleost enzymes was undertaken by comparative reciprocal western blot analysis. Cross-reactivity between teleost and mammalian antibodies further supported a close structural as well as functional similarity between teleost and human CYP3A enzymes (Celander et al., 1996; Miranda et al., 1991). Functional characterization of recombinant CYP3A enzymes has been determined for CYP3A27, CYP3A45, CYP3A38, and CYP3A40 by heterologous expression in baculovirus systems. Recombinant rainbow trout CYP3A27 exhibited a maximum CO-reduced spectrum at 450 nm and comigrated with purified CYP3A27 (formerly denoted LMC5) on western blots. In reconstitution experiments, recombinant protein exhibited catalytic activities for the 6β-, 2β-, and 16β-hydroxylation of

TABLE 4.4

CYP3 Phylogeny in Fishes

Species	Common Name	CYP3A Gene	Accession Number	Immunodetection	Detection/ Localization	Refs.
Oncorhynchus mykiss	Rainbow trout	CYP3A27	O42563	Pos 1, 2	L, IC, PDI, B, INV	Lee et al. (1998)
Fundulus heteroclitus	Killifish	CYP3A30	AF105068	Pos 1, 2	L, K, I, G, S, B, O	Celander and Stegeman (1997)
Oryzias latipas	Medaka	CYP3A38	AF105018	Pos 2	Liver microsomes	Kullman et al. (2000)
		CYP3A40	AF251272	Pos 2	Liver microsomes	Kullman and Hinton (2001)
Oncorhynchus mykiss	Rainbow trout	CYP3A45	AF267126	Pos 1, 2	IC, PDI, INV (low liver)	Szklarz and Halpert (1997)
Fugu rubripes	Pufferfish	CYP3A48	PNC	—	—	—
		CYP3A49	PNC	—	—	—
		CYP3A50P (pseudogene)	PNC	—	—	—
Fundulus heteroclitus	Killifish	CYP3A56	AY143428	Pos 1	L, K, I, G, S, B, O	Hegelund and Celander (2003)
Danio rerio	Zebrafish	CYP3A65	PNC	—	—	—
Micropterus salmoides	Largemouth bass	CYP3A68	PNC	—	—	—
		CYP3A69	PNC	—	—	—
Platichthys flesus	European flounder	CYP3A (partial cds)	AJ310471	Pos 1, 2	Liver microsomes	Celander et al. (1996b); Williams et al. (2003)
Ctenopharyngodon idella	Carp	CYP3A	AAL16897	Pos 1	—	Lao et al. (unpublished)
Fugu rubripes	Pufferfish	CYP3B1	PNC	—	—	—
		CYP3B2	PNC	—	—	—

Danio rerio	Zebrafish	CYP3C1	PNC	—	—	—
Fugu rubripes	Pufferfish	CYP3D1 (was 3A47)	PNC	—	—	—
Gadus morhua	Atlantic cod	—	—	Pos 1, 2	Liver microsomes	Hasselberg et al. (2004)
Oreochromis niloticus	Tilapia	—	—	Pos 1, 2	Liver microsomes	Pathiratne and George (1996)
Salmo salar	Atlantic salmon	—	—	Pos 1	Liver microsomes	Arukwe et al. (1997)
Salvelinus fontinalis	Brook trout	—	—	—	—	Celander et al. (1996b)
Pleuronectes platessa	Plaice	—	—	Pos 1, 2	Liver microsomes	Celander et al. (1996b)
Salmo trutta	Brown trout	—	—	Pos 1, 2	Liver microsomes	Celander et al. (1996b)
Scophthalmus maximus	Turbot	—	—	Pos 1, 2	Liver microsomes	Arukwe and Goksøyr (1997); Celander et al. (1996b)
Kyphosus sectatrix	Bermuda chub	—	—	Pos 1, 2	Liver microsomes	Stegeman et al. (1997)
Haemulon sciurus	Blue-striped grunt	—	—	Pos 1, 2	Liver microsomes	Stegeman et al. (1997)
Chaetodon capistratus	Butterfly fish	—	—	Pos 1, 2	Liver microsomes	Vrolijk et al. (1994)
Haemulon aurolineatum	Tomtate	—	—	Pos 1, 2	Liver microsomes	Stegeman et al. (1997)
Tinca tinca	Tench	—	—	—	B, G, L Gi, H	Klinger et al. (2001)
Salvelinus alpinus	Arctic char	—	—	Pos 1	Liver microsomes	Jorgensen et al. (2001)
Abudefduf saxatilis	Sergeant major	—	—	Pos 1, 2	Liver microsomes	Stegeman et al. (1997)
Anguilla anguilla	Eel	—	—	Pos 1	Liver microsomes	Van der Oost et al. (2001)
Poeciliopsis lucida	Hepatocellular cell line (PLHC-1)	—	—	Pos 1, 2	—	Celander et al. (1996a)

Note: 1, recognized by polyclonal antibodies raised against trout LMC5 or P450con; 2, recognized by polyclonal antibodies raised against scup P450A; PNC, cytochrome P450 nomenclature committee; B, brain; G, gut; Gi, gill; I, intestine; IC, intestinal ceca; INV, intestine near vent; K, kidney; L, liver; O, ovary; PDI, proximal descending intestine; S, spleen.

testosterone at 1.48, 0.043, and 0.034 nmol/min/nmol, respectively, as well as dehydrogenation of nifedipine at 50 pmol/min/nmol (Lee and Buhler, 2002). Although turnover rates were significantly higher with the recombinant enzyme, hydroxylase profiles were similar to that observed with purified CYP3A27 enzyme. Heterologous expression of the rainbow trout paralog CYP3A45 additionally exhibits testosterone hydroxylase activity with 6β-hydroxytestosterone as the major metabolite. Activity was higher than that observed with CYP3A27 and was significantly enhanced by the addition of cytochrome b_5 to the reconstitution assays (Lee and Buhler, 2003).

Recombinant medaka CYP3A38 and CYP3A40 catalyzed hydroxylation of testosterone, as well as the *O*-debenzylation of benzyloxyresorufin (BR) and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC); however, efficiencies and specificities were significantly different between the two isoforms. Thus, K_m and V_{max} activities based on BFC *O*-debenzyloxylase were estimated to be 0.116 and 0.363 μ*M* and 7.95 and 7.77 nmol/min/nmol P450 for CYP3A38 and CYP3A40, respectively. Medaka CYP3A38 preferentially catalyzed testosterone hydroxylation at the 6β- and 16β-positions, with minor hydroxylation at other positions within the steroid nucleus, whereas CYP3A40 catalysis was predominantly limited to the 6β- position (Kashiwada, unpublished data). Putative identification of CYP3A SRS1 to SRS6 indicated that 12 of the 49 amino acid differences between CYP3A38 and CYP3A40 occur in SRS1, SRS3, and SRS5, previously known to be associated with steroid hydroxylation (Kashiwada, unpublished data; Kullman and Hinton, 2001). Functional analysis of teleost and mammalian CYP3A paralogs has demonstrated that gene-duplication events are tied to acquisition of new function and that convergent evolution of CYP3A function may be frequent among independent gene copies (McArthur et al., 2003). The physiological role of CYP3A is yet to be determined. Given the robust steroid hydroxylase activity, there is speculation that CYP3A forms play an important part in steroid (hormones as well as bile acids) homeostasis. Although the physiological function of CYP3A is still unknown, the fact that these genes are expressed in tissues that act as barriers to the environment (i.e., digestive and respiratory tracts) together with the broad substrate specificities of CYP3A enzymes suggest that they evolved as biochemical defense to prevent bioaccumulation of xenobiotics. In addition, the presence of CYP3A enzymes in steroidogenic tissues implies a role in steroid biotransformation.

Regulation—CYP3A are the major constitutive CYP forms expressed in the liver and intestine of most mammals and other species, including fish (Celander et al., 1996, 1989; Hegelund and Celander, 2003; Hegelund et al., 2004; Husoy et al., 1994). Numerous studies have demonstrated, however, that constitutive expression of CYP3A genes between species, and paralogous CYP3A forms within species, are highly variable with age, gender, development, tissue localization, and between individuals. Variations in gene expression may be due to external environmental factors, including temperature, salinity, diet, or other environmental stressors; biological factors, such as circulating hormone levels; or tissue-specific factors associated with development or reproductive cycle. Depending on species, sexual dimorphic expression of CYP3A has been observed. Lee et al. (1998) reported higher levels of CYP3A expression in juvenile female rainbow trout intestine when compared to males. Gender differences were additionally observed in kidney and liver but to a lesser degree than in intestine. A similar trend was demonstrated in winter flounder (*Pleuronectes americanus*) hepatic microsomes and in Atlantic cod, where CYP3A expression was sevenfold higher in females than males (Gray et al., 1991; Hasselberg et al., 2004). In contrast, adult killifish males displayed up to 2.5-fold higher levels of hepatic and extrahepatic CYP3A30 and CYP3A56 mRNA and protein levels compared to females (Hegelund and Celander, 2003). This finding was in agreement with higher hepatic testosterone 6β-hydroxylase activities in male killifish (Gray et al., 1991; Stegeman and Woodin, 1984). Other species, including medaka and sexually mature rainbow trout, also exhibit a higher level of CYP3A expression in male liver microsomes compared to females (Aoyama et al., 1990; Celander et al., 1989, 1996; Gillam et al., 1993; Gotoh, 1992; Harlow and Halpert, 1998; Kullman and Hinton, 2001; Lee and Buhler, 2003; Li et al., 1995; Smith et al., 1996; Szklarz and Halpert, 1997; Waxman et al., 1998). Variations in CYP3A expression in Atlantic salmon and turbot (*Scophthalmus maximus*) during reproductive cycles have been reported, implying a regulatory role of sex steroids on CYP3A expression (Arukwe and Goksøyr, 1997; Larsen et al., 1992). Steroid reproductive hormones and plasma growth hormones have been shown to influence the sexual dimorphic expression of CYP3A in rodents (Park et al., 1999; Sakuma et al., 2002; Wang and Strobel, 1997).

In teleosts, the effects of exposure to reproductive hormones on CYP3A expression differ depending on species. Numerous studies with rainbow trout, brook trout, medaka, and other fish species have demonstrated that exposure to 17β-estradiol results in a suppression of total microsomal CYP content. This correlated with decreased CYP3A mRNA expression, CYP protein levels, or steroid hydroxylase activities (Buhler et al., 2000; Celander et al., 1989; Pajor et al., 1990). Treatment of male Atlantic cod with 17β-estradiol resulted in an increase in CYP3A protein expression (Hasselberg et al., 2004). The mechanisms of hormonal regulation of CYP genes in teleosts have yet to be determined; however, it is possible that circulating hormone levels (steroids or growth hormone) are associated with sexual dimorphic differences in CYP expression in fish.

Numerous pharmaceuticals and xenobiotics have been demonstrated to alter CYP3A gene transcription in mammals via binding and transactivation of members of the nuclear receptor family NR1, including the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the vitamin D receptor (VDR) (Pascussi et al., 2003; Plant and Gibson, 2003). As mentioned above, although Maglich et al. (2003) did not find CAR in the pufferfish genome, they identified a single PXR/CAR gene that was more PXR like, indicating CAR may have evolved after divergence of fish or may have been lost in some or all teleosts. To date, PXR and VDR have been identified in few teleost species. Prototypical mammalian NR1 (PXR) receptor agonists, including dexamethasone (DEX), rifampicin (RIF), and pregnenolone-16α-carbonitrile (PCN), are seemingly less effective at altering teleost CYP3A gene transcription (Celander et al., 1989, 1996a,b; Kullman, unpublished data). In tilapia, PCN treatment resulted in twofold induction of CYP3A proteins (Pathiratne and George, 1996). The ligand-binding region of PXR was isolated from zebrafish (Kliewer et al., 2002). This region was shown to be activated by some prototypical mammalian PXR agonists, including nifedipine, phenobarbital, clotrimazole, and some steroids, but PCN, DEX, and RIF did not activate the fish receptor (Moore et al., 2002). Induction of hepatic CYP3A expression *in vivo* by ketoconazole was observed in juvenile rainbow trout and Atlantic cod (Hasselberg et al., 2004; Hegelund et al., 2004). As mentioned above, response to 17β-estradiol resulted in either enhanced or diminished CYP3A expression, depending on the species examined (Buhler and Wang-Buhler, 1998; Hasselberg et al., 2004; Husoy et al., 1994; Pajor et al., 1990). Furthermore, xenoestrogens also affect CYP3A expression. Male Atlantic cod exposed to alkylphenols showed enhanced hepatic CYP3A expression, whereas treatment with ethinylestradiol and nonylphenol suppressed CYP3A expression in Atlantic salmon and Atlantic cod (Arukwe et al., 1997; Hasselberg et al., 2004). Slight increases in hepatic CYP3A expression have been demonstrated in rainbow trout exposed to cortisol, whereas cortisol treatment had no effect on CYP3A protein levels in Arctic char (*Salvelinus alpinus*) (Celander et al., 1989; Jorgensen et al., 2001).

Although gene sequences for PXR and VDR have been identified in fish (Kliewer et al., 2002), few functional data exist regarding their role in transcriptional activation of CYP3 genes. Identification of cognate hormone response elements upstream of pufferfish and medaka CYP3A genes suggest that nuclear receptors may be involved in transcriptional regulation; however, this has yet to be determined (Kullman, pers. commun.). Cloning and analysis of lamprey VDR suggest that this nuclear receptor is capable of binding and transcriptional activation of the mammalian CYP3A4-DR3 hormone response element. These results demonstrate conservation in the DNA binding behavior of an early form of this receptor and a possible role in transcriptional activation of CYP genes in fish. (For more on nuclear receptors in teleosts, see Chapter 7). Modulation of teleost CYP3A expression following xenobiotic exposure is highly variable and species dependent.

Studies examining the binding behavior of mammalian PXR have demonstrated transactivation and CYP3A induction by xenoestrogens, including bisphenol A, nonylphenol, DDT, and other organochlorine pesticides (Courmoul et al., 2002; Masuyama et al., 2000; Takeshita et al., 2001; You, 2004). The response of aquatic species to these and other PXR, CAR, and VDR ligands has been minimal; thus, the overall induction of CYP3A in teleosts appears to be considerably weaker than that reported in mammals. Compared to mammalian species, this suggests that teleost CYP3A expression may be governed by alternative transcriptional mechanisms. Given the recent completion of several teleost genome projects, examination of promoter sequences will shed some light on regulatory elements and transcriptional control of theses genes. In several studies, discrepancies have been noted between changes in gene expression and changes in steroid hydroxylase activity. In a study by Hasselberg et al. (2005), exposure

of juvenile Atlantic cod to ketoconazole resulted in slight increases in CYP3A expression but marked decreases in CYP3A-specific catalytic activity. Similar discrepancies have been noted in medaka, for which significant decreases in catalytic activity cannot be accounted for by corresponding reductions in CYP3A expression (Kashiwada, unpublished data). As noted in the section on CYP1A, these discrepancies between message level and catalytic activity necessitate determination of expression at several levels prior to making conclusions about regulatory mechanisms.

Localization—Localization of CYP3A expression is similar to that observed in mammalian species, with high levels of expression found in the liver and intestinal mucosa. Some differences are observed between specific CYP3A paralogs and may represent putative regulatory and functional differences that occurred during gene duplication events. Immunohistochemical and mRNA analysis of CYP3A27 has demonstrated strong responses in intestinal ceca, proximal descending intestine, and liver, with minor expression occurring in brain (Cok et al., 1998; Lee et al., 2001). The rainbow trout paralog CYP3A45 was predominantly expressed in the gastrointestinal tract, with weak expression occurring in the liver (Lee and Buhler, 2003). In killifish, CYP3A30 and CYP3A56 were coexpressed in intestine and liver, consistent with that observed for rainbow trout CYP3A27. In both species, CYP3A expression is prominent in the digestive tract (intestinal mucosa and liver), suggesting a role for these enzymes in first-pass metabolism of xenobiotics. Extrahepatic CYP3A expression is observed in gill, kidney, brain, spleen, and ovary, suggesting a possible role for CYP3A enzymes in the fine-tuning of endogenous substrates at the site of synthesis or action as well as metabolic defense against xenobiotics (Hegelund and Celander, 2004).

Development—Biotransformation systems for embryonic and fetal tissues of vertebrates are not as well characterized as those for adults. Embryogenesis is a dynamic process that presents a continuously changing metabolic profile as enzymes are induced and repressed (Miller et al., 1996). In general, developing organisms lack many CYP forms present in adults; however, in some instances, they contain certain forms that are only expressed during development. These differences in total CYP content may explain developmental processes and altered sensitivities to toxic exposures during development. Teleost embryos are often sensitive to xenobiotics, and prolonged exposures result in numerous developmental malformations, early mortality, and delayed hatch (Cooke and Hinton, 1999; Villalobos et al., 1996). The nonspecific onset of many of these aberrations may reflect an inability of embryos to sufficiently metabolize and detoxify many of these xenobiotics. CYP3A27 cDNA was cloned from both embryonic and adult hepatic mRNA, suggesting this gene is expressed early during rainbow trout development (Lee et al., 1998). Kullman and Hinton (2001) demonstrated that CYP3A38 and CYP3A40 genes are differentially regulated during embryonic development. Analysis of CYP3A38 and CYP3A40 mRNA and protein demonstrated the presence of a single CYP3A transcript for early and late embryonic stages and two CYP3A transcripts in larvae and adult liver in medaka. Using gene-specific probes, results demonstrated that CYP3A40 is expressed early in embryonic development and continues throughout adult stages. CYP3A38, however, is tightly suppressed during embryonic development and is only expressed post-hatch. Given the role of CYP enzymes in maintaining steady-state levels of morphogenic ligands, it is not surprising to find these enzymes in the earliest stages of life. Previous studies have provided strong evidence for the presence of multiple forms of CYP in the developing fish embryo (Buhler et al., 1997; Chen and Cooper, 1999). In general, fetal CYP forms are present in low levels and exhibit stringent temporal expression patterns that diminish following birth or hatching (Juchau et al., 1998). CYP3A40 mRNA expression was detected as early as stage 11, representing an early multicellular stage of development. Liver formation does not occur until stage 26, demonstrating that initial CYP3A40 expression occurs prior to organogenesis. Functionally, expression of CYP3A40 during early embryonic development may serve multiple purposes including xenobiotic metabolism, hydroxylation of steroid or other morphogenic ligands, and metabolism of yolk. As with human CYP3A7, CYP3A may be the only constitutively expressed cytochrome P450 during embryogenesis, although some adults do express CYP3A7.

Mechanisms of Stimulation and Inhibition—Mutation and docking studies have demonstrated that CYP3A proteins have a large substrate-binding pocket in comparison to other members of the CYP superfamily (Khan and Halpert, 2000). This large pocket is thought to enable CYP3A enzymes to bind

multiple substrate molecules at any given time and results in unusual kinetic behaviors consistent with allosteric interaction. The atypical kinetic behavior of CYP3A enzymes results in either sigmoidal or convex rate–substrate concentration profiles indicative of positive or negative cooperativity (Houston and Kenworthy, 2000). With human CYP3A4, homotropic cooperativity (i.e., one substrate) has been observed with numerous chemicals and results in an initial lag in the rate–substrate concentration profile, thus generating sigmoidal profiles (Harlow and Halpert, 1997). Addition of secondary substrates such as α-naphthoflavone (ANF) can result in modification of testosterone hydroxylation activity, suggesting heterotropic (two or more substrates) cooperative interaction (Harlow and Halpert, 1997, 1998). Activation of BFC *O*-debenzyloxylase activity by ANF also was observed in rainbow trout liver microsomes (Hegelund and Celander, unpublished data). In studies with CYP3A38 and CYP3A40, addition of higher (>25 μ*M*) BFC concentrations resulted in a decrease of catalytic activity and a convex rate–substrate plot. This type of deviation from Michaelis–Menten kinetics is indicative of negative homotropic cooperativity and is due to an inability to maintain V_{max} at higher substrate concentrations. In separate experiments, nonylphenol was used as a heterotropic effector. Results were biphasic, suggesting that nonylphenol acts as a concentration-dependent cooperative activator and inhibitor of BFC catalysis. The rate substrate plot demonstrates a sigmoidal curve at low concentrations (below 100 n*M*), indicative of heterotropic activation; however, at higher concentrations, activity levels are decreased, resulting in a convex rate–substrate plot that is indicative of heterotropic inhibition (Kullman et al., 2004). Rainbow trout and killifish exposed to ketoconazole, a potent antifungal agent, additionally exhibited significant decreases in CYP3A-mediated BFC *O*-debenzyloxylase activity, suggesting that this pharmaceutical is a potent heterotropic inhibitor of CYP3A activity (Hegelund et al., 2004). Alkylphenol additionally inhibited CYP3A activity in Atlantic cod liver microsomes with an IC_{50} of 100 μ*M* compared to ketoconazole, which had an IC_{50} in a submicromolar range (Hasselberg et al., 2004). These studies demonstrate that teleost CYP3A enzymes exhibit unusual kinetic behaviors consistent with allosteric interaction and cannot be described by hyperbolic kinetic models. Homotropic cooperative inhibition of BFC at high concentrations suggests that the CYP3A protein is capable of binding multiple substrate molecules, which may result in autoactivation or inhibition of catalysis. Interestingly, the addition of nonylphenol, alkylphenol, or ketokonazole results in heterotropic cooperative inhibition at environmentally relevant concentrations (Hasselberg et al., 2004; Hegelund et al., 2004). Given the putative role of CYP3A in maintaining the homeostatic balance for numerous endobiotics, enzymatic activation and inhibition by xenobiotic compounds may represent a (nongenomic) mechanism of altered metabolism and subsequent toxicity.

Flavin-Containing Monooxygenases

Overview

Flavin-containing monooxygenases (FMOs) are a multigene family of enzymes involved in the monooxygenation of primarily soft-nucleophilic-heteroatom-containing compounds (see Table 4.5) and some inorganic compounds (Hines et al., 1994; Ziegler, 1988; Ziegler and Mitchell, 1972). Because FMOs are located in the smooth endoplasmic reticulum (microsomes) and require NADPH and oxygen as cofactors for catalysis, FMO-catalyzed reactions were thought at one time to merely be another mixed-function oxidase reaction. FMOs were finally identified as unique enzymatic entities following purification of the enzyme by Ziegler and Mitchell (1972). Through an elegant series of experiments, Poulsen and Ziegler (1979) showed that FMOs have a distinct reaction mechanism that is not dependent on a reductase coenzyme (such as CYP), but they are directly reduced by NADPH. The reduction of the flavin by NADPH then allows binding of molecular oxygen, creating a hydroperoxyflavin that is very susceptible to nucleophilic attack by soft-nucleophilic-heteroatom-containing compounds and various inorganic species; thus, compounds with non-delocalized electronic features, such as tertiary amine- and sulfur-containing compounds, are excellent substrates for FMOs (Ziegler, 1988; Ziegler and Mitchell, 1972). Numerous substrates have been identified in mammalian systems, but few have been examined in fish (Schlenk, 1993, 1998).

One of the most surveyed, but more difficult to measure, FMO-catalyzed reactions in fish is that of trimethylamine oxidase (or more accurately TMA oxygenase) (for reviews, see Baker et al., 1963; Schlenk, 1998). Although FMO has yet to be purified to homogeneity in fish, numerous inhibition and

TABLE 4.5

Putative Substrates for Flavin-Containing Monooxygenases in Fish

Substrate	Product
Nitrogen-containing	
Trimethylamine	Trimethylamine *N*-oxide
N,*N*-Dimethylaniline	*N*,*N*-Dimethylaniline *N*-oxide
2-Aminofluorene	2-Hydroxyaminofluorene[a]
Sulfur-containing	
Thiobencarb	Thiobencarb-*S*-oxide[a]
Thiourea	Thiourea-sulfonic acid[a]
Methimazole	Methimazole-sulfonic acid[a]
Aldicarb	Aldicarb sulfoxide[a]
Selenium-containing	
Dimethylselenide	Dimethylselenoxide[a]
Selenomethionine	Selenomethione-Se-oxide[a]

[a] More toxic metabolite than parent compound.

correlative studies have indicated that TMA oxidase is catalyzed by FMO (Agutsson and Strom, 1981; Goldstein and Dewitt-Harley, 1973; Peters et al., 1995; Schlenk, 1994; Schlenk and Li-Schlenk, 1994; Schlenk et al., 1995). A direct relationship also exists between TMA content in fish tissues and enzyme expression (Larsen and Schlenk, 2001; Raymond, 1998; Raymond and DeVries, 1998; Schlenk, 1998). Fish that possess high tissue (muscle, liver, blood) concentrations of TMA or TMA *N*-oxide have higher levels of expression and enzyme activity than fish that do not have either of these biomolecules; for example, TMA-lacking species, such as the channel catfish (*Ictalurus punctatus*), do not express FMO-like protein or enzymatic activity, whereas TMA-containing rainbow trout or various elasmobranchs possess relatively high levels of FMO activity and expression in various tissues (Schlenk and Buhler, 1991; Schlenk and Li-Schlenk, 1994; Schlenk et al., 1993). The relationship between TMA and FMO has several implications regarding the evolution and possible physiological functions of these enzymes which are discussed further below.

Xenobiotics that have been shown to be substrates of the enzyme in fish include tertiary amines such as *N*,*N*-dimethylanaline (DMA) and TMA, thioether pesticides (e.g., aldicarb), thiocarbamates (e.g., eptam, thiobencarb), thiocarbamides (e.g., methimizole, thiourea), and thioamides (e.g., thiobenzamide) (Table 4.5); for a review, see Schlenk (1998). Currently, the most characterized diagnostic substrates for FMO activity in fish are thiourea and DMA (Schlenk, 1993, 1998). Enzymatic activities in fish appear to be sensitive to temperature and have a relatively high pH optimum of 8.0 to 9.6 in various fishes (Schlenk, 1993, 1998). Each of these assays is a simple spectrophotometric method; however, other more elaborate high-performance liquid chromatography (HPLC) methods utilizing enantioselective oxygenations have been identified in mammals to differentiate activities catalyzed by specific isoforms (Rettie et al., 1995). Recent studies have indicated unique stereochemical sulfoxidation reactions in trout, which have not been observed with any previous isoforms of mammalian FMOs (Schlenk et al., 2004). Typically, to validate FMO activity for an uncharacterized xenobiotic, co-incubation of other FMO substrates or CYP inhibitors is necessary, because FMO and CYP share many substrates. It is imperative to note, however, that many putative inhibitors of mammalian enzymes are not as effective in fish and few have been well-characterized, so care should be taken when interpreting data regarding the use of enzyme inhibitors (especially CYP and FMO) in studies with fish.

Currently, 12 FMO genes have been identified in mammals (classified as FMO1 to FMO12), but none has been fully characterized in any fish species (Hines et al., 1994). A close examination of accessible genomic sequences of the pufferfish (*Fugu* sp.) has indicated a gene fragment that is 42% identical with FMO4 and 56% identical to FMO5 (Dolphin, pers. commun.). Conservation of the secondary structure of FMOs has been observed in fish using western blot analyses that employed antibodies raised against mammalian forms (El-Alfy and Schlenk, 1998; Peters et al., 1995; Schlenk, 1998; Schlenk and Buhler,

1993; Schlenk and Li-Schlenk; Schlenk et al., 1995). At least two microsomal proteins in rainbow trout liver were recognized by antibodies raised against porcine FMO1 (Schlenk and Buhler, 1993). Subsequent studies with antibodies raised against human FMO1 only recognized one of the two bands (Schlenk et al., 2004). Although a faint band was noted in Japanese medaka liver microsomes with *anti*-FMO3, a stronger signal from *anti*-FMO1 was observed (El-Alfy and Schlenk, 2002); however, catalytic activities (stereochemical analyses) in trout and shark do not correspond to FMO1 or FMO3 but seem to resemble FMO4-like activities, which tends to support genetic observations in *Fugu* (Schlenk et al., 2004).

Regulation, Function, and Toxicological Relevance

Regulation of FMO expression appears to be extremely complex, with the enzyme often being expressed in a random manner (Baker et al., 1963). As mentioned earlier, FMO has been observed in all marine fish species, some euryhaline, and virtually no freshwater species. Strong correlations have been observed between FMO1-like proteins, enzymatic activity, and mRNA recognized by FMO1 cDNA in juvenile Atlantic flounder (*Platichthys flesus*) and turbot (*Scophthalmus maximus*) (Peters et al., 1995; Schlenk et al., 1996a,b), but catalytic activity did not correspond with mRNA expression in sexually mature adult flounder (Schlenk, unpublished data). In fact, although FMO1-like mRNA was observed in all sexually mature animals, hepatic FMO activity was lacking in more than 40% of the animals, with males having more frequent expression. Although evidence suggests that various hormones may modulate the expression of FMO (El-Alfy and Schlenk, 2002; El-Alfy et al., 2002; Schlenk et al., 1997), no consistent induction of enzyme expression has been observed following xenobiotic treatment. In medaka, estradiol downregulated the hepatic FMO3-like protein but induced the gill FMO1-like form (El-Alfy and Schlenk, 2002). Testosterone downregulated both forms of FMO and activity in medaka. Arterial infusion of cortisol induced expression of FMO1 in rainbow trout gill and liver (El-Alfy et al., 2002). However, infusion of growth hormone failed to alter FMO activity or expression (Schlenk, unpublished data).

As mentioned above, FMO activity and protein in several tissues, particularly the gill, appear to be directly correlated with serum osmolality or the salinity regime in which the fish resides (Daikoku and Sakaguchi, 1990; El-Alfy and Schlenk, 2002; Schlenk, 1998; Schlenk and El-Alfy, 1998; Schlenk et al., 1996a,b). Several hypotheses have been put forth to explain this relationship. Four possibilities are: (1) TMA *N*-oxide is produced as a cellular defense to prevent the enzyme inactivation by high cellular ion (Na, K) or urea that occurs in fish residing in subartic or subantarctic environments (Raymond, 1998; Raymond and DeVries, 1998); (2) TMA *N*-oxide is produced to counterbalance high tissue and serum levels of urea that are present regardless of temperature (e.g., in sharks) (Van Waarde, 1988; Yancey et al., 1982); (3) TMA *N*-oxide may be produced in muscle of deep-sea gadiform teleosts (which also produce urea) as an adaptive defense against high pressure (Gillett et al., 1997); and (4) TMA *N*-oxide may be formed by FMOs as a secondary organic osmolyte in response to shifts in salinity regimes (Schlenk, 1993). Each scenario is interrelated, with the common similarity being hyperosmolality. In some euryhaline fish, FMO activity and expression are directly related to the salinity regime in which the animal resides (Charest et al., 1988; Daikoku and Sakaguchi, 1990; Daikoku et al., 1988; El-Alfy and Schlenk, 2002; Lange and Fugelli, 1965; Schlenk and El-Alfy, 1998; Schlenk et al., 1996a,b). FMO activity and protein expression is higher in gills and kidneys than liver in several species of euryhaline fish such as the Atlantic flounder (*Platichthys flesus*), Japanese medaka (*Oryzias latipes*), and rainbow trout (Larsen and Schlenk, 2001; Schlenk, 1998; Schlenk and El-Alfy, 1998; Schlenk et al., 1995). In addition, FMO activity in trout and medaka is downregulated following steroid treatment, which also downregulates osmoregulatory function (i.e., Na^+/K^+-ATPase) (McCormick, 1995; Schlenk et al., 1997). FMO activity and expression in rapid osmoconformers such as striped bass and tilapia do not respond to changes in salinity (Wang et al., 2001), which is also consistent with mechanisms of Na^+/K^+-ATPase regulation in these species. Testing scenarios 1 and 3, recent studies in rainbow trout have indicated that urea infusion or reductions in temperature induce FMO activity and increase muscular TMA *N*-oxide concentrations (Larsen and Schlenk, 2001). Given induction following cortisol treatment, several factors may be involved in FMO regulation in rainbow trout, including stress resulting from alterations in cellular redox potential due to urea or hyperosmotic conditions (El-Alfy et al., 2002; Larsen and Schlenk, 2001). Clearly, more studies are necessary to better understand the regulation of this enzyme system given its role in xenobiotic biotransformation and toxicology.

The toxicological significance of FMO-catalyzed biotransformation reactions has not been extensively examined in fish. Recent studies with organoselenides and FMO from sharks have indicated that FMO may be involved in the oxidation and initiation of redox cycling in these species (Schlenk et al., 2003); however, most studies examining the toxicological roles of FMO have examined pesticides. The oxygenation of the pesticides thiobencarb and eptam in striped bass (*Morone saxatilis*) by hepatic FMO was shown to lead to the formation of a reactive-intermediate that covalently bound protein sulfhydral groups (Cashman et al., 1990; Perkins et al., 1999); however, protein binding was not observed *in vivo* by thiobencarb. *S*-Oxygenation of aldicarb to the sulfoxide by FMO significantly increased the inhibition of acetylcholinesterase in rainbow trout (*Oncorhynchus mykiss*) (250-fold) and Japanese medaka (*Oryzias latipes*) (40-fold) (El-Alfy and Schlenk, 2002; Perkins et al., 1999). Elevated toxicity has been observed in FMO-containing fish which can activate aldicarb to the more potent sulfoxide compared to species that lack FMO and convert aldicarb to the less toxic sulfone or hydrolytic metabolites (Perkins and Schlenk, 2000; Schlenk, 1995).

Enhanced sulfoxidation may possibly explain the enhanced toxicity of aldicarb in higher salinity observed in medaka and trout, as FMO expression has been shown to be directly correlated to salinity in medaka (Larsen and Schlenk, 2001; Schlenk and El-Alfy, 1998). Studies comparing the effects of salinity on aldicarb toxicity in trout and striped bass indicate that salinity significantly enhances the toxicity of aldicarb in trout but not in striped bass (Wang et al., 2001). In striped bass, aldicarb sulfoxide formation and FMO expression were unchanged by salinity, whereas salinity increased aldicarb sulfoxide formation, cholinesterase inhibition, and FMO expression in rainbow trout. Consequently, understanding factors that affect the expression patterns of FMO is important when considering species-specific sensitivities to xenobiotics and differential responses of organisms to environmental factors such as salinity and temperature regimes.

Monoamine Oxidases

Monoamine oxidases catalyze the oxidation and eventual elimination of alpha carbon groups from secondary amines. Monoamine oxidases have been characterized in several fish species, with most occurring in trout. Given the critical importance in catecholamine metabolism, most studies have focused on its endogenous role in the neurophysiology of fish. In contrast to terrestrial vertebrates, which have two forms of the enzyme (MAO A and MAO B), fish appear to only have a single form that is genetically distinct from terrestrial vertebrates. Although no specific studies have examined the role of MAO in xenobiotic biotransformation in fish, the effects of various organic and inorganic pollutants on enzyme activity has been examined (Senatori et al., 2003).

Alcohol and Aldehyde Dehydrogenases

Alcohol dehydrogenase (ADH) catalyzes the oxidation of alcohols to aldehydes, which are subsequently converted to acids by aldehyde dehydrogenase (ALDH). NAD^+ is a cofactor for each enzyme. A class 3 ADH cDNA was first identified in sea bream, in which its expression was observed in all tissues as well as eggs and embryos. Expression decreased during early embryonic development but increased fourfold from day 1 to 21 after hatching, indicating that the maternal ADH mRNA is present in the eggs and embryos but diminishes as development occurs, allowing the larval tissue to express its own ADH (Funkenstein and Jakkowiew, 1996). An additional ADH3 cDNA was also identified by RT-PCR in zebrafish (*Danio rerio*) (Dasmahapatra et al., 2001). Expression of the gene in zebrafish embryos appeared to correspond with temporal variations in zebrafish susceptibility to ethanol toxicity. In cod, an ADH enzyme was purified that displayed structural similarities to ADH3, but functionally it was more like ADH1. Ethanol was an excellent substrate for the purified enzyme, and 4-methylpyrazole was a strong inhibitor ($K_i = 0.1\ \mu M$) (Danielsson et al., 1992). Allylic and acetylenic alcohols appear to be bioactivated through oxidation by trout liver ADH and may also act as inhibitors (Bradbury and Christensen, 1991). ALDH has been observed in all tissues of numerous fish species (Nagai et al., 1997). Similar to CYP1A, ALDH has been observed in mammals to be regulated by the Ah receptor. Studies in the dab (*Limanda limanda*), the sea bass (*Dicentrarchus labrax*), and the rainbow trout failed to observe increases in ALDH activities following 3-MC or BNF treatment (Le Maire et al., 1996); however, expression was significantly elevated in liver tumor tissues from adult rainbow trout treated with aflatoxin (Parker et al., 1993).

Peroxidases

Lipoxygenases, cyclooxygenases, and peroxidases such as prostaglandin-H synthetase are typically involved in the oxidation of arachidonic acid to hydroxy and peroxyeicosatetraenoic acids, which are subsequently converted to prostaglandins, prostacylins, and thromboxanes. In mammals, each of these enzymes has been shown to activate various xenobiotics through cooxidation pathways requiring a hydroperoxide as a cofactor. Although the enzymes have been observed in fish, their contributions to xenobiotic biotransformation has not been examined. Examples of lipoxygenases observed in rainbow trout gills include the 15- and 12-lipoxygenase (German and Berger, 1990; Hsieh et al., 1988). PGH synthetase has also been observed in gills of freshwater fish (Christ and Van Dorp, 1972).

Aldehyde Oxidase

Aldehyde oxidase is a molybdozyme that is similar to xanthine oxidase located in the cytosols of liver in mammals and fish. Pyrroles, pyridines, pyrimidines, purines, and aromatic aldehydes derived from catecholamine metabolism tend to undergo oxidation reactions; however, under anaerobic conditions, 2-hydroxypyrimidine, N^1-methylnicotinamide, or butyraldehyde may act as electron donors in liver cytosols in fish, leading to substrate reduction under these conditions. Enzyme activity is inhibited by menadione, β-estradiol, and chlorpromazine. Recent studies in the goldfish (*Carassius auratus*) and the sea bream (*Pagrus major*) have demonstrated that aldehyde oxidase catalyzes the reduction of fenthion sulfoxide to the parent sulfide (Kitamura et al., 2003).

Reductases

One of the most common reductases involved in xenobiotic biotransformation is the cytochrome P450 reductase, which catalyzes single-electron reductions to substrates prone to accept single electrons (e.g., quinones, cyclic or aromatic amines). Often, this pathway activates heterocyclic compounds to redox cycling intermediates. Limited studies have evaluated the role of this enzyme in biotransformation in fish.

DT Diaphorase

DT diaphorase (NAD(P)H:[quinone acceptor] oxidoreductase) plays a significant role in one- and two-electron reduction reactions, particularly in the liver. 4-Nitroquinoline 1-oxide and nitrofurantoin were both reduced by DT diaphorase to genotoxic metabolites in a brown bullhead fibroblast cell line (Hasspieler et al., 1997). A novel dicoumarol-sensitive oxidoreductase that catalyzes the reduction of phenanthrenequinone was purified from gastric cytosol in the channel catfish (*Ictalurus punctatus*) (Hasspieler and Di Giulio, 1994). Due to its likely AhR-mediated regulation, the enzyme has shown promise as a biomarker in fish (see Chapter 16). Whether or not bifunctional regulation through ARE occurs (as in mammals) is uncertain.

Azo- and Nitroreductases

Catalyzing up to three sequential two-electron reductions, nitroreductases play a significant role in the biotransformation of primarily nitroaromatic compounds to corresponding amino aromatic compounds. When 2-nitrofluorene was incubated with liver microsomes or cytosol of sea bream (*Pagrus major*) in the presence of NADPH or 2-hydroxypyrimidine, 2-aminofluorene was formed (Ueda et al., 2002). Hepatic nitroreductases were also observed in catfish and catalyzed the formation of superoxide from nitrofurantoin, *p*-nitrobenzoic acid, and *m*-dinitrobenzene (Washburn and Di Giulio, 1988). Hepatic nitro- and azoreductases were observed in the marine teleosts barracuda (*Sphyraena barracuda*) and yellowtail snappers (*Ocyurus chrysurus*); however, only azoreductase activity was observed in elasmobranches, such as the lemon shark (*Negaprion brevirostris*) and stingray (*Dasyatis americana*) (Adamson et al., 1965). The lampricide 3-trifluoromethyl-4-nitrophenol was reduced by nitroreductases to 3-trifluoromethyl-4-aminophenol in rainbow trout (Lech and Costrini, 1972).

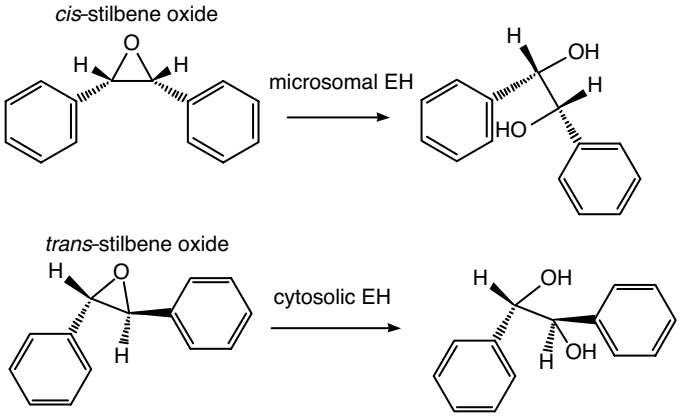


FIGURE 4.5 Reactions catalyzed by epoxide hydrolases.

Hydrolysis

Epoxide Hydrolase

Overview

Xenobiotic epoxides and arene oxides are usually formed by cytochrome P450-dependent oxygenation of a double bond or an aromatic ring. Due to the strain of the three-membered oxirane ring, they readily react with cellular nucleophiles such as water, glutathione, or nucleophilic centers in DNA bases. The function of the epoxide hydrolase (EH) group of enzymes is to catalyze the addition of water to an epoxide or arene oxide. Epoxide hydrolase enzymes are considered part of a larger class of hydrolytic enzymes, including esterases, proteases, dehalogenases, and lipases (Beetham et al., 1995). Studies with mammalian enzymes have shown that two major epoxide hydrolase enzymes utilize xenobiotic epoxides as substrates. These are the cytosolic enzyme that utilizes *trans*-epoxides as substrates and the microsomal enzyme that prefers *cis*-epoxides and arene oxides. In both cases, the products are *trans*-dihydrodiols (Hammock and Hasagawa, 1983) (see Figure 4.5). In a given animal, no evidence indicates multiple forms of the major microsomal or cytosolic epoxide hydrolase.

Microsomal epoxide hydrolase is of particular importance for arene oxides produced by the action of CYP on polycyclic aromatic hydrocarbons. For most arene oxides, conversion to the dihydrodiol results in detoxification of the PAHs. In some cases, however, epoxide hydrolase plays a role in the formation of reactive diol epoxide metabolites; for example, conversion of benzo(*a*)pyrene-7,8-oxide to the 7,8-dihydrodiol is part of the pathway leading to the ultimate carcinogen, (+)-*anti*-benzo(*a*)pyrene-7,8-dihydrodiol-9,10-oxide.

The preferred substrates for study of microsomal epoxide hydrolase activity are *cis*-stilbene oxide (shown in Figure 4.5) and benzo(*a*)pyrene-4,5-oxide, both of which are commercially available in radiolabeled form. The earliest studies of this enzyme were conducted with racemic styrene oxide, but this epoxide was thought to be less definitive in measuring microsomal epoxide hydrolase activity than a true *cis*-epoxide. Regardless of substrate, the method most commonly used to measure epoxide hydrolase activity was to incubate the radiolabeled epoxide or arene oxide with microsomes at pH 9 and then measure the amount of product formed. The pH optimum of microsomal EH activity in most species that have been examined was 8.5 to 9.5 (Balk et al., 1980; James et al., 1979). For some substrates (e.g., styrene oxide and *cis*-stilbene oxide), unreacted substrate was separated from product by extraction (Gill et al., 1983; James et al., 2004) and for others, such as benzo(*a*)pyrene-4,5-oxide, by chromatography (Jerina and Dansette, 1977). Other methods have also been developed, such as gas chromatography, spectrophotometric, and fluorimetric methods (Dansette et al., 1976; Westkaemper and Hanzlik, 1981), although these tend to be of lower sensitivity than the radiochemical methods. In all methods, an important consideration is to keep the reaction pH 6 or higher at all steps, as epoxides undergo spontaneous hydrolysis at acidic pH.

TABLE 4.6

Microsomal Epoxide Hydrolase Activity with Styrene Oxide in Representative Fish Species

Species	Organ	Activity[a]	Refs.
Channel catfish (*Ictalurus punctatus*)	Liver	2.62 ± 1.0	James et al. (1997); Willett et al. (2000)
	Intestine	3.78 ± 1.95	
	Liver	1.03 ± 0.06	
Brown bullhead (*Ameiurus nebulosus*)	Liver	0.85 ± 0.07	Willett et al. (2000)
Trout (*Oncorhynchus mykiss*)	Liver	1.2	Perdudurand and Cravedi (1989)
Sturgeon (*Acipenser baeri*)	Liver	2.77	Perdudurand and Cravedi (1989)
	Kidney	1.37	
	Gill	1.37	
Sheepshead (*Archosargus probatocephalus*)	Liver	5.6 ± 2.4	James et al. (1974)
	Kidney	0.05	
	Intestine	0.74 ± 0.33	
	Gill	0.16	
	Ovary	0.08	
	Testis	0.52	
Southern flounder (*Paralichthyes lethostigma*)	Liver	2.0 ± 0.7	James et al. (1974)
	Kidney	1.33 ± 0.11	
Winter flounder (*Pseudopleuronectes americanus*)	Liver	27.9 ± 15.9	James et al. (1974)
	Kidney	8.25 ± 6.11	
Northern pike (*Esox lucius*)	Liver	4	Balk et al. (1980)
English sole (*Parophrys vetulus*)	Liver	2.8 ± 0.2	Collier et al. (1986)
Scup (*Stenotomus chrysops*)	Liver	2.6–9	Stegeman and James (1985)
Dogfish shark (*Squalus acanthias*)	Liver	6.3 ± 2.1	James et al. (1974)
	Kidney	12.6 ± 3.9	
Atlantic stingray (*Dasyatis sabina*)	Liver	6.2 ± 1.8	James et al. (1974)
	Kidney	3.1 ± 1.8	
Large skate (*Raja ocellata*)	Liver	1.8 ± 0.2	James et al. (1974)
	Kidney	0.55	
Small skate (*Raja erinacea*)	Liver	0.46 ± 0.4	James et al. (1974)
	Kidney	0.16	

[a] In units of nmol · min^{-1} · mg $protein^{-1}$.

In mammalian species, antibodies have been used to quantitate microsomal epoxide hydrolase enzyme in liver subcellular fractions (Bend et al., 1978). Such studies have not been conducted in fish. The cross-reactivity of fish epoxide hydrolases with mammalian microsomal or cytosolic epoxide hydrolases is not known, and no fish epoxide hydrolase protein or gene has yet been reported.

Enzyme Specificity, Regulation, and Inhibition

The first studies of epoxide hydrolase activity in fish were carried out with hepatic, branchial, gonadal, and intestinal microsomes from marine fish common to coastal Maine or Florida (Bend et al., 1978; Gill et al., 1982; James et al., 1974, 1976; Westkaemper and Hanzlik, 1981). These studies showed that, although epoxides were readily hydrolyzed in liver of most fish species, considerable variability exists between individuals as well as between species in the measured epoxide hydrolase activity. Gill, kidney, intestine, and gonads generally had lower epoxide hydrolase activity than liver. Freshwater fish also had readily measured epoxide hydrolase activity in liver and other organs (Parker et al., 1993; Perdudurand and Cravedi, 1989; Stott and Sinnhuber, 1978; Walker et al., 1978; Willett et al., 2000). Table 4.6 shows activities found in some representative marine and freshwater species in liver and other organs.

The importance of epoxide hydrolase activity in the biotransformation of polycyclic aromatic hydrocarbons was highlighted in a study of the *in vitro* metabolism of benzo(*a*)pyrene in scup hepatic microsomes (Stegeman and James, 1985). The ratio of BaP-9,10-dihydrodiol to 9-hydroxy-BaP was found to correlate well with the epoxide hydrolase activity of hepatic microsomes. Individual fish with low epoxide hydrolase

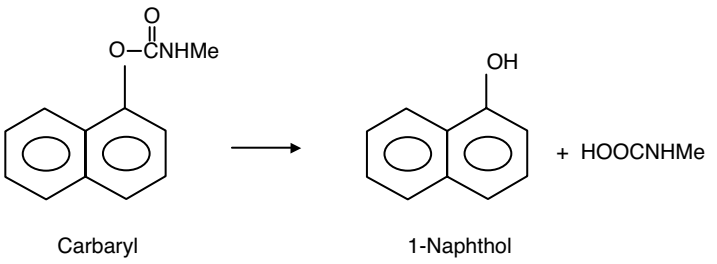


FIGURE 4.6 Reactions catalyzed by carboxylesterases.

activity formed proportionally more of the phenolic rearrangement product, 9-hydroxy-BaP, than BaP-9,10-dihydrodiol; individuals with high epoxide hydrolase activity formed proportionally more BaP-9,10-dihydrodiol and less of the phenolic rearrangement product. This relationship was not found for the ratio of BaP-7,8-dihydrodiol to 7-hydroxy-BaP. It was thought that this was because of differences in the chemical reactivity of the arene oxides of BaP. BaP-9,10-oxide, unlike BaP-7,8-oxide, was not readily hydrolyzed in the absence of epoxide hydrolase, making the presence of the epoxide hydrolase enzyme critical to determining if BaP-9,10-oxide would be hydrolyzed to the dihydrodiol or rearrange to the 9-hydroxy product. The presence of compounds that modulate enzyme activity was found to influence the metabolites of BaP formed in hepatic microsomes from control or 3-methylcholanthrene-induced sheepshead (Little et al., 1981). Incubation of BaP with hepatic microsomes in the presence of naphthoimidazole, a substance that inhibits CYP1A but stimulates epoxide hydrolase, gave a higher ratio of BaP-9,10-dihydrodiol to 9-hydroxy-BaP than incubations in the absence of the modulating agent, presumably by routing the BaP-9,10-oxide formed by CYP1A to BaP-9,10-dihydrodiol rather than to 9-hydroxy-BaP.

Modulation of epoxide hydrolase activity has been investigated following treatment with various xenobiotics. In mammalian species, several xenobiotics induce epoxide hydrolase activity, including phenobarbital, *trans*-stilbene oxide, and some aryl hydrocarbon receptor agonists, including PCBs (Bresnick et al., 1977; Gillette et al., 1987). In fish, however, induction of epoxide hydrolase activity following administration of these agents has not been demonstrated (James and Little, 1981; James et al., 1997). Indeed, in stingrays treated with 3-methylcholanthrene at a dose that induced AHH activity tenfold, epoxide hydrolase activity was significantly lower in hepatic microsomes from treated fish (4.54 ± 0.55 nmol/min/mg protein) relative to controls (5.81 ± 0.76) (James and Bend, 1980). A similar trend was observed in 3-MC-treated sheepshead. Flatfish exposed to PAH- and PCB-contaminated Puget Sound sediments showed no increase in epoxide hydrolase activity (Collier and Varanasi, 1991; Collier et al., 1986). Likewise, channel catfish and brown bullhead treated with 10 mg/kg benzo(*a*)pyrene showed no significant induction or species difference in liver microsomal *cis*-stilbene-oxide hydrolase activities (Willett et al., 2000). In the splake, treatment with the fish anesthetic tricaine methane sulfonate reduced epoxide hydrolase activity in liver and duodenum (Laitenen et al., 1981).

Carboxylesterases

Hydrolytic biotransformation of xenobiotics by various forms of carboxylesterases in fish plays a significant role in the detoxification of various pesticides (Glickman et al., 1982; Straus and Chambers, 1995; Wallace and Dargan, 1987) and plasticizers (Barron et al., 1989) (Figure 4.6). Most of the studies examining this pathway in fish have focused on postmitochondrial or cytosolic enzymes (Salamastrakis and Haritos, 1988), and some studies have examined the microsomal activities (Soldano et al., 1992; Vittozzi et al., 2001). No carboxylesterase genes have been characterized in fish, but studies with purified proteins (e.g., chlorpyrifos) have been carried out (Boone and Chambers, 1997). Differences in carboxylesterase activities among species have been hypothesized to be responsible for the acute toxicity of organophosphates (Keizer et al., 1991, 1993, 1995) and pyrethroids (Glickman et al., 1979, 1982). Carp were resistant to diazinon toxicity because of relatively high activity of hydrolyzing esterase activity, whereas trout was very sensitive to toxicity because of a lack of esterase activity and a sensitive acetylcholinesterase (Keizer et al., 1995). A similar relationship was observed in trout with permethrin (Glickman et al., 1979).

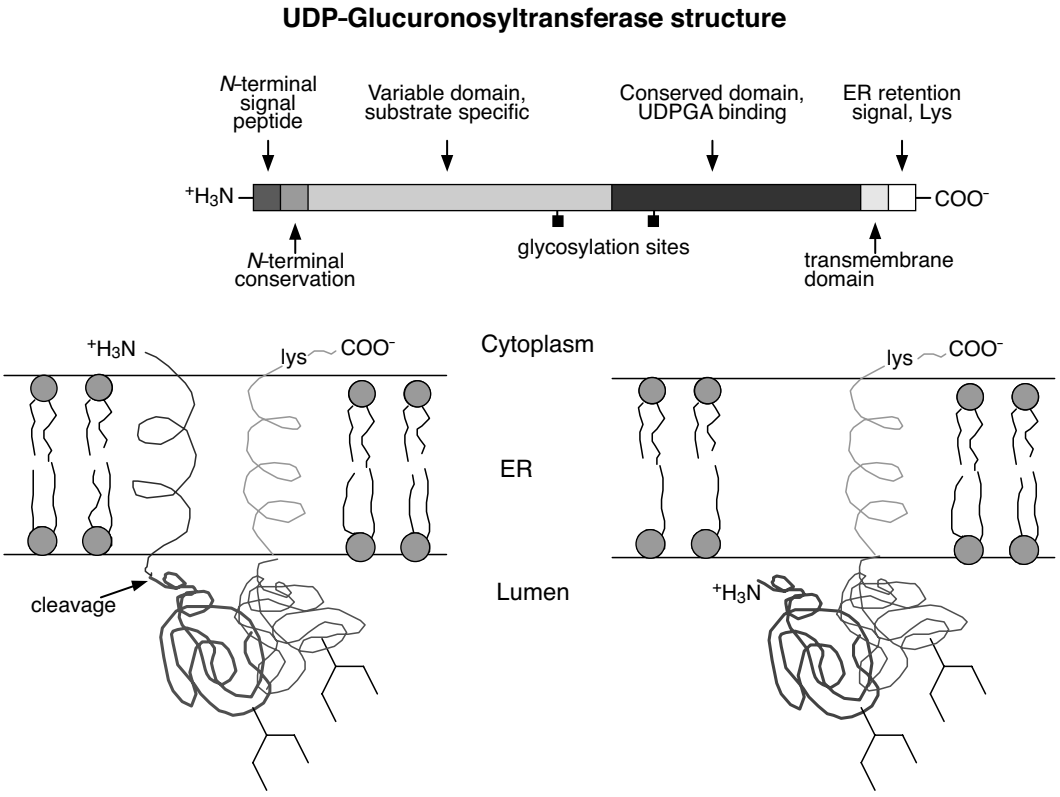


FIGURE 4.7 Structure and organization of UDP–glucuronosyltransferases.

Phase II Enzymes

UDP-Glucuronosyltransferases

Overview

The UDP-glucuronosyltransferases (UGTs) represent a major group of phase II conjugating enzymes. Glucuronidation is principally a characteristic feature of vertebrates; invertebrates tend to prefer to utilize glycosylation. Glucuronidation is the major pathway for the conversion (and inactivation) of both endogenous and exogenous compounds to polar, water-soluble compounds that are then excreted in the bile (compounds > 350 MW) or urine (compounds < 300 MW). The UGTs are active in the metabolism of endogenous compounds such as steroid hormones, thyroid hormones, and waste products such as bilirubin (Dutton, 1990). In addition, an important role is played in the biotransformation of natural toxins and anthropogenic toxicants that are absorbed into the organism. Because of their importance in the breakdown of therapeutic drugs, UGTs are extensively researched in the medical and pharmaceutical fields (Parkinson, 2001). This explains why so much is known about UGTs in mammalian systems, while research on UGTs in lower vertebrates and invertebrates is much more limited.

The glucuronosyltransferases are located in the endoplasmic reticulum (ER), with the active site facing inward into the lumen of the ER (Figure 4.7). The various isozymes have a common *C*-terminal, which anchors the enzyme in the membrane of the ER. This brings them in close proximity to phase I enzymes, such as CYP1A, which are also located on the ER; thus, phenolic phase I metabolites formed by CYPs can immediately be conjugated by the neighboring phenol-type UGT (UGT1A6). It has even been suggested that direct contact between CYP1A1 and UGT1A6 occurs and that these protein–protein interactions enhance the activity of the glucuronidation enzyme (Taura et al., 2004).

Glucuronidation involves the transfer of an activated sugar group to the substrate. This process increases the water solubility and the molecular weight of the substrates, thus facilitating transport and excretion. The preferred cofactor for most UGT isoforms is uridine diphosphoglucuronic acid (UDPGA). However, a number of other nucleotide–sugar cofactors have been reported for these enzymes; therefore, the name *UDP-glycosyltransferase* has been proposed as a better name for this superfamily (Mackenzie et al., 1997). UDPGA is synthesized in the cytosol from uridine-triphosphate and glucose-1-phosphate. Contrary to many phase I reactions, glucuronidation does not require additional energy supply in the form of ATP or NADPH. The synthesis of UDPGA in the cytosol introduces an extra threshold for the glucuronidation reaction rate, as the water-soluble cofactor has to be actively transported through the ER membrane to reach the active site on the UGT enzyme. Mechanisms surrounding transport are still unknown.

UGT Gene Structure

Multiple UGT genes have been characterized in mammals (17 in humans) and are subdivided into two families. All UGT1 family members are encoded by a unique single locus gene, which in humans has 13 different first exons spanning some 300 kb. They encode the aglycone binding site, which is alternatively spliced onto identical exons 2 to 5, which encode the *C*-terminal half of the protein responsible for UDP-glucuronic acid binding. Each exon 1 appears to have its own promoter in the 5′ upstream region between its transcription start site and the preceding exon 1. Elaboration of the gene appears to have arisen from duplication into two clusters: (1) a *bilirubin cluster* (1A1 to 1A5 isoforms), primarily involved in the conjugation of amines, bilirubin, carboxylic acids, and thyroxine, and (2) a *phenol cluster*, which conjugates planar polyaromatic hydrocarbons (1A6) and bulky phenols (1A7 to 1A13). A similar although slightly less complex elaboration of the UGT1A family has been found in rodents and the rabbit. In humans and rats, at least one gene of the bilirubin cluster is a pseudogene resulting in reduced conjugation of planar phenols. In contrast, the genes of UGT2 family members, each comprised of six exons, are located at distinct loci on another chromosome. The UGT2A subfamily has postulated roles in olfaction, and the products of the UGT2B family genes appear to be particularly involved in bile acid and steroid hormone conjugation. Although many of the enzymes were successfully purified from rodents, the majority of the genes have been heterologously expressed for activity studies, and indeed most of the human enzymes are now commercially available.

The gene encoding the major phenol-conjugating isoform of plaice has been cloned (UGT1B1) and found to share an almost identical five-exon structure with mammalian UGT1 family members (George and Leaver, 2002; George, unpublished data). When heterologously expressed, it displays specificity for planar phenols such as 1-naphthol and 4-nitrophenol as aglycones but no demonstrable activity with bilirubin or steroids (Leaver et al., 2007).

Expressed sequence tags (ESTs) for UGTs have subsequently been cloned from flounder, plaice, and zebrafish. Analysis of the currently published zebrafish EST sequences and the latest assembly of the genome reveals that at least 14 distinct UGTs are expressed (George and Taylor, 2002; George, unpublished data, Leaver et al.., 2007). More putative genes can be identified in the genome; however, they are found on numerous chromosomes (in both mouse and humans, the UGT1 family occurs on one chromosome and the UGT2 family on another). The phylogenetic relationships between these expressed zebrafish UGT homologs and the human genes is shown in Figure 4.8. The zebrafish genes are divisible into three groups; the group with closest similarity to the characterized UGT1B1 gene comprises two alternatively spliced six-exon genes. Gene 6220 is comprised of six exon 1's, three of which are known to be expressed (Figure 4.9). The products arise by alternative splicing of the primary transcripts. Inspection of the nucleotide sequences of the intervening introns between the multiple exon 1's reveals the presence of peroxisomal proliferator response elements (PPREs) and xenobiotic response elements (XREs), which would support the plaice induction data. A further three zebrafish genes are comprised of six exons and divide with the mammalian UGT2 family, thus inferring the closest homology. Interestingly, gene 4649 also appears to exhibit alternative splicing. Intronless UGT genes can also be identified in zebrafish (four putative genes, two reported as ESTs) and in the pufferfish (two genes). These genes (family 3) have an unknown function and appear to be unique to fish.

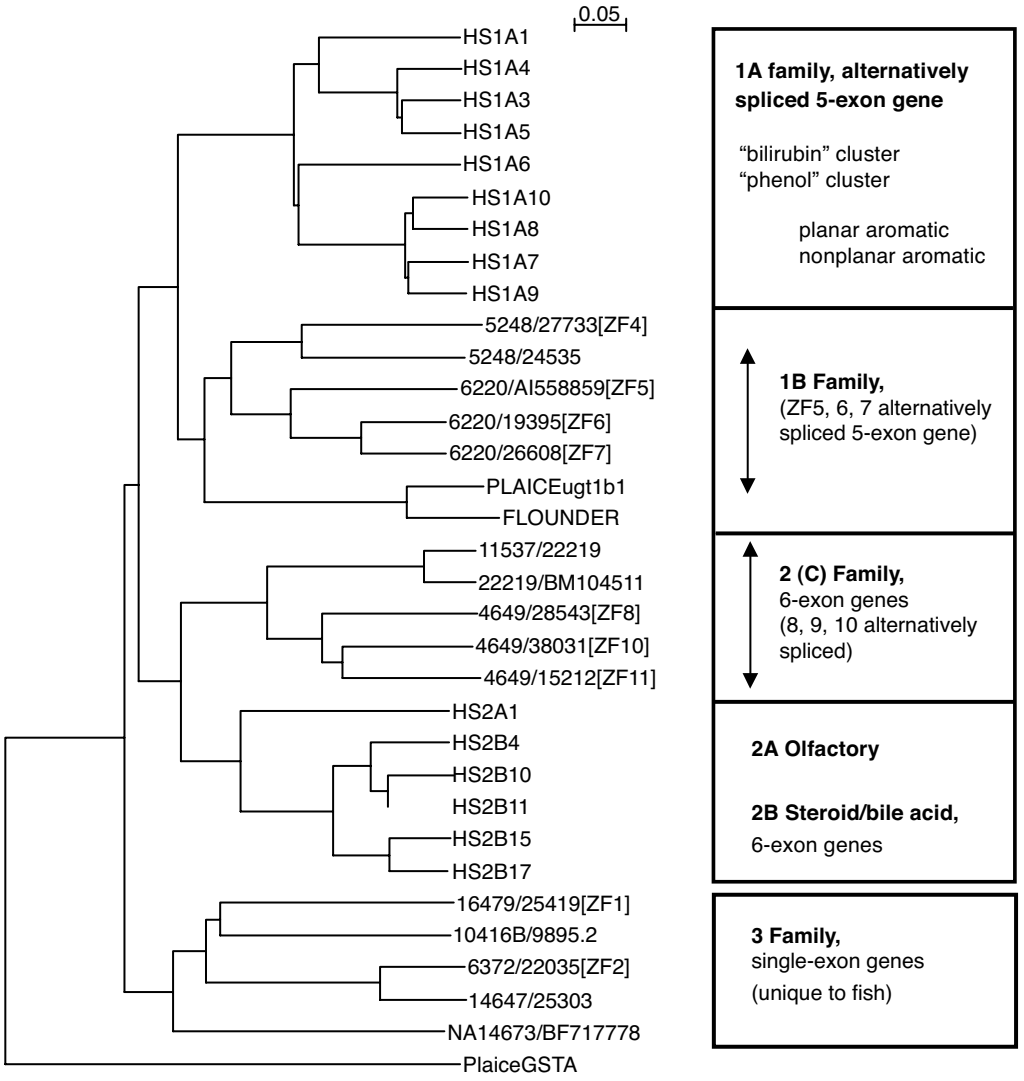


FIGURE 4.8 Phylogenetic relationships of expressed fish and human (HS) UGTs. The zebrafish protein sequences were translated from the genome sequence for which there are reported EST data (gene/protein numbers given [ZF]). (From George, S.G. and Taylor, A., *Mar. Environ. Res*., 54, 253, 2002. With permission.)

Reactions and Substrate Specificity

The spectrum of the acceptor substrates is very wide and, as with most enzymes that exhibit a broad specificity for structurally diverse compounds, multiple isoenzymes belonging to a number of multigene families are found (Mackenzie et al., 1997). The enzymes catalyze the transfer (conjugation) of glucuronic acid from the high-energy nucleotide UDP-glucuronic acid (UDPGA) to a wide variety of acceptor substrates (aglycones) to form β-glucuronides (Figure 4.10). The most common are the formation of *O*-glucuronides from alcohols, phenols, and carboxylic acids and *N*-glucuronides of carbamates, amides, and amines. *S*-Glucuronides of aryl mercaptans and thiocarbamates and *C*-glucuronides formed by conjugation of 1,3-dicarbonyls have also been identified in mammals but not so far in aquatic species. The endogenous roles of these enzymes are in the detoxification of toxic metabolites (e.g., bilirubin) formed from the degradation of heme, the secretion of bile acids (e.g., lithocholate), termination of hormone action (retinol, T_3, T_4, sex steroids), cessation of the action of olfactory stimulants, and, in the

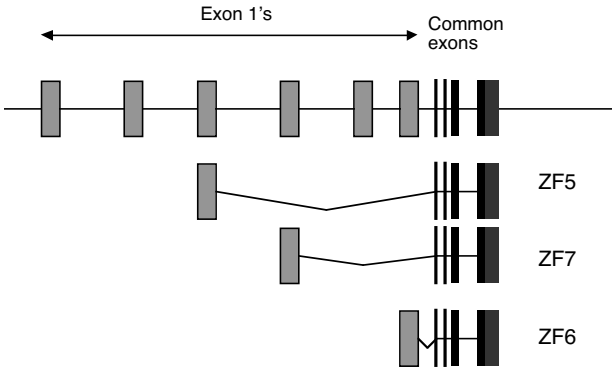


FIGURE 4.9 Structure of the zebrafish UGT1B gene showing alternative splicing of primary transcripts.

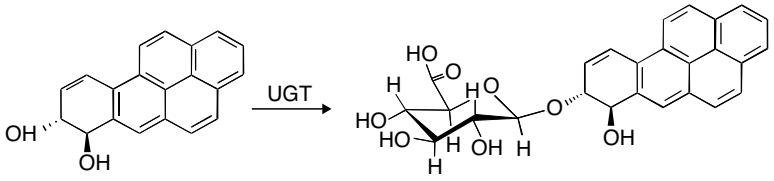


FIGURE 4.10 Glucuronidation of benzo(*a*)pyrene-7,8-dihydrodiol catalyzed by UGT.

case of fish, excretion of glucuronides of sex steroids as pheromones and spawning stimulants (Lambert and Resink, 1991). Many of the enzymes have a broad and overlapping substrate specificity, which gives them the ability to conjugate many xenobiotic compounds in addition to their preferred endobiotic substrates. This appears to be the case for those conjugating bilirubin and planar polyaromatic phenols, although several isoenzymes, particularly those metabolizing steroid hormones, appear to show a more restricted substrate specificity (Table 4.7).

Most investigations of fish have been *in vivo* studies concerned with the identification of the glucuronides of endobiotic and xenobiotic compounds. Glucuronide conjugates of bilirubin, 17β-estradiol, triiodothyronine (T_3), and thyroxine (T_4) have been identified in the bile of several fish species, whereas glucuronides of 3α-, 3β-, and 17β-hydroxy steroids and their metabolites have been identified in several tissues and body fluids, showing that the scope for endogenous compounds is the same as in mammals (George, 1994).

Quantitatively, glucuronidation is the most important pathway for detoxication and excretion of xenobiotic compounds in mammals, and studies to date indicate that this is also the case in fish. A recent listing of the wide and structurally diverse range of xenobiotic compounds whose glucuronides have been detected in bile, urine, and various tissues is given in Table 4.8. Compared with the large number of compounds shown to be glucuronidated *in vivo*, few studies have been reported on the scope of

TABLE 4.7

Substrate Specificities of Some Mammalian UGT Isoenzymes and Some Xenobiotics Conjugated

Endogenous Substrates	**Xenobiotic Substrates**
Bilirubin, thyroxine, 5-OH tryptamine	4-Nitrophenol (4NP)
Retinol (?)	1-Naphthol (1-NA)
Testosterone (17β-OH steroid)	4-Methyl umbelliferone
Androsterone (3α-OH steroid), lithochlolate	4-Aminobiphenyl
Estrone, 2-estradiol	
Olfactory stimulants	

TABLE 4.8

Compounds Glucuronidated in Fish

Chemical Class	Examples
Aromatic hydrocarbons	Benzene (metabolite phenol) Naphthalene (metabolite 1-naphthol)
Aliphatic hydrocarbons	Hexachlorocyclohexane (lindane)
Polyaromatic hydrocarbons (phenol and diol metabolites)	Phenanthrene, pyrene, chrysene, benzo(*a*)pyrene, retene (7-isopropyl-1-methylphenanthrene)
N-Heteroaromatics	Quinoline, dimethylquinoline, carbazole
Aromatic amines	Aniline, 2,4-dichloroaniline, naphthylamine
Thioazole	2-Amino-4-phenylthiazole (anesthetics, such as Piscaine™; the *N*-glucuronide)
O-Heteroaromatics (hydroxylated or dealkylated metabolites)	Dibenzofuran(s), tetrachlorodibenzofuran, 7-ethoxycoumarin
S-Heteroaromatics	Dibenzothiophene
Biphenyls (hydroxylated metabolites)	Biphenyl, tetrachlorobiphenyl
Resin acids	Abietic, hehydroabietic, hydroabietic, isopimaric, pimaric acids (present in wood pulps)
Phenolics	Phenol 1-naphthol, 4-amino phenol, 1-chlorophenol, *penta*-chlorophenol (wood preservatives), chlorophenolics formed during paper bleaching, 4-nitrophenol, 3-trifluoromethylnitrophenol (lampreycide), phenolphthalein, phenolsulfonphthalein (dyes, slow), aflatoxicol M (aflatoxin B_1 metabolite)
Phenolic xenoestrogens	Bisphenol A, diethylstilbesterol, 4-nonylphenol, nonylphenol diethoxylate, *tert*-octylphenol (degradation products of alkylphenoxylate detergents)
Phytoestrogens	Coumesterol, genistein, biochinin A
Antibiotics	Chloramphenicol, oxolinic acid, dimethylquinoline, miloxacin
Insecticides	Organophosphates (e.g., fenitrothion malathion, chloropyriphos); carbamates (e.g., 1-naphthyl-*N*-methylcarbamate, Sevin™); pyrethroids (e.g., pyrethrin)
Fungicides	Imidazole (e.g., Prochloraz™), pentachlorophenol
Plasticizers	*mono*-Ethylhexylphthalate, di-2-ethylhexylphthalate
Miscellaneous industrial chemicals, drugs	Picric acid, picramic acids, morphine, valproic acid, pristane, digoxigenin monodigitoxide
Endobiotics	Bilirubin, bilirubin glucuronide; cholic acids, cholate, deoxycholate, lithocholate; retinoic acid; triodothyronine (T3), thyroxine (T4); 3α-hydroxysteroids (androsterone); 3-hydroxysteroids (estradiol, estrone); 17β-hydroxysteroids (testosterone, 17-methyltestosterone)

glucuronidation in isolated microsomal preparations from fish. The liver is the most active tissue (see later section). Planar phenols such as 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone are readily conjugated, although the rate may vary by as much as an order of magnitude between species. One difficulty in intercomparison is the well-known latency observed in microsomes due to the lumenal orientation of the enzyme and inaccessibility of UDPGA. In fish, this latency, which varies in a tissue-specific manner, does not appear to be so great as in mammals. Maximal activity in microsomes is only obtained in the presence of an optimized amount of detergent; indeed, an excess of detergent has an inhibitory effect (Burchell and Coughtrie, 1989; Clarke et al., 1992b). Many published results are not comparable because the species differences are remarkable, most notable being the much lower capacity for conjugation of phenols in trout than plaice (George, 1994).

Enzymology of Piscine UGTs

The UGTs are membrane-bound enzymes that are quite labile when isolated, requiring phospholipids to maintain activity; therefore, UGTs have proven to be notoriously difficult to purify and characterize. The only non-mammalian UGTs to be purified were from the plaice (Clarke et al., 1992c). At least six immunoreactive UGT peptides were visualized in plaice microsomes in western blots with mammalian

antisera, indicating common structural epitopes (Clarke et al., 1992a). From purification studies, at least five UGTs have been identified in plaice liver (George, 1994), and the phenol-conjugating isoform that was purified to homogeneity displayed a very high activity toward planar phenols and no measurable activity toward bilirubin or steroids. The bilirubin-conjugating isoform appeared to exhibit both bilirubin- and phenol-conjugating activity as found in mammals. Reviews of earlier studies on glucuronidation in fish can be found in Clarke et al. (1991) and George (1994). The picture that emerges from these reviews is that UGT isoforms are found in a wide variety of fish species and that specific enzymatic activities are found for bilirubin, steroid and thyroid hormones, and phenolic compounds. As in mammalian systems, a diverse group of xenobiotic compounds has been identified as substrates for UGTs, among which are chlorinated phenols, aromatic hydrocarbon metabolites, phthalates, aflatoxin, pesticides, and antibiotics. Comparison of UGT activities among species was found to be a problematic task primarily due to the non-optimization of detergent concentrations in assays in most published studies. The enzymes also display maximal activity at 37°C (Clarke et al., 1992b); thus, corrections for temperature should be used for intercomparisons.

Tissue Distribution

In fish species, UGT activity is usually high in liver and intestine, but measurable activities have also been found in gill, kidney, and muscle tissue (Clarke et al., 1991; George et al., 1998; James et al., 1998; Singh et al., 1996). UGTs play an important role in gonadal tissues; in addition to the regulation of steroid hormones, UGTs play a role in the production of sex pheromones in fish (Lambert and Resink, 1991). Testosterone UGT activity is present in liver, testis, and intestine (Clarke et al., 1992b), and glucuronidation of pregnenolone and androstenedione has been demonstrated *in vitro* with testicular preparations (Andersson, 1992). Glucuronidation of bilirubin is confined to liver in both plaice and salmon.

Regulation of UGTs

Coregulation of both CYPs and UGTs occurs in mammals, and prototypical inducers such as clofibrate, PAHs, phenobarbital, and pregnenolone-16α-carbonitrile (PCN) differentially induce expression of both CYP and UGT isoforms. The degree of upregulation of UGT activity is generally some two- to threefold. UGT1A1 (bilirubin conjugation) is induced by the hyperlipidemic agent (and peroxisomal proliferator) clofibrate. In common with a number of phase I and II genes, UGT1A6 (planar phenol conjugation) is induced by interaction of the Ah receptor with an XRE in its promoter region. This coregulation of CYP1A and UGT1A6 in mammals and CYP1A and UGT1B1 in fish has been shown to facilitate detoxification of PAHs such as BaP. Mammalian CYP2B and CYP3A and UGT1A genes are also induced via a nuclear pregnane X receptor and a constitutive androstane receptor. Interestingly, these recognition motifs are also present in the zebrafish UGT6220 gene (George, unpublished data). Mammalian steroid UGTs are induced by PCN. Several reports indicate a modest induction of phenol UGT activity in fish from polluted environments and after experimental PAH exposure. To study the induction of AhR-activated enzymes, two PAHs are often used: 3-methylcholanthrene (3-MC) and β-naphthoflavone (BNF). Both compounds have been used as CYP1A and phenol UGT inducers in a number of fish species (Table 4.9). Maximal induction of CYP1A is usually found around 3 days after single treatment, but phenol UGT induction appears to be slower, with a maximum induction occurring around 8 days after treatment. In general, induction of EROD activity can be up to 250-fold, but UGT activity is never induced more than 3-to 6-fold. Two species, cod (*Gadus morhua*) and gilthead sea bream (*Sparus aurata*), displayed little or no response to AhR ligands as phenol UGT inducers (Goksøyr et al., 1987; Pretti et al., 2001). It must be noted, however, that multiple isoforms in mammals, including the constitutive steroid isoforms, also conjugate 4-nitrophenol. Immunoblot and northern blot analyses of xenobiotic-treated plaice have shown that induction of UGTs appears to be tissue specific (Clarke et al., 1992a). Treatment with the PAH (3-MC) increased phenol-conjugating activity and a 56-kDa immunoreactive peptide in liver by approximately 1.7-fold. The bifunctional PAH-type inducer BNF caused an induction (approximately three- to fourfold) of UGT1B1 mRNA only in intestine (Leaver et al., unpublished data). Although clofibrate did not appear to induce phenol- or bilirubin-conjugating activities (Clarke et al., 1992a),

TABLE 4.9

Effects of β-Naphthoflavone (BNF) and 3-Methylcholanthrene (3-MC) on UGT Induction in Various Fish Species

Species	Inducer	Dose	Maximum Induction	Ref.
Rainbow trout (*Oncorhynchus mykiss*)	BNF	100 mg/kg	3×	Andersson et al. (1985)
	BNF	50 mg/kg	2×	Celander and Forlin (1995)
	BNF	5 mg/kg	1.7×	LeMaire et al., 1996
Sea bass (*Dicentrarchus labrax*)	3-MC	20 mg/kg	1.5×	LeMaire et al., 1996
	BNF	80 mg/kg	1.8×	Novi et al. (1998)
	BNF	0.3–0.9 μ*M* aqueous	3×	Gravato and Santos (2002)
Dab (*Limanda limanda*)	3-MC	20 mg/kg	No induction	LeMaire et al., 1996
Plaice (*Pleuronectes platessa*)	3-MC	10 mg/kg	1.4×	George and Young (1986)
Channel catfish (*Ictalurus punctatus*)	3-MC	50 mg/kg	1.7×	Gaworecki et al. (2004)
Brown bullhead (*Ictalurus nebulosus*)	3-MC	20 mg/kg	2×	Pangrekar and Sikka (1992)
Mummichog (*Fundulus heteroclitus*)	3-MC	50 mg/kg	1.7–2×	Gaworecki (unpublished data)

TABLE 4.10

Effects of PCBs on the Induction of UGTs in Various Fish Species

Species	Inducer	Dose (mg/kg)	Maximum Induction	Ref.
Rainbow trout (*Oncorhynchus mykiss*)	PCB 77, 126	1–5	1.6×	Huuskonen et al. (1996)
	Clophen A50	100	2×	Forlin et al. (1996)
	3,3′,4,4′-TCB	0.3	1.75–2×	Blom and Forlin (1997)
Brook trout (*Salvelinus fontinalis*)	3,3′,4,4′-TCB	10	1.5×	Boyer et al. (2000)
Sand flathead (*Platycephalus bassensis*)	Arochlor® 1254	400	1.75×	Brumley et al. (1995)

treatment with the potent peroxisome proliferator-activated receptor γ (PPARγ)-mediated peroxisomal proliferator perfluorooctanoate (PFOA) has not been found to induce UGT1B1 mRNA levels in tissues other than kidney (Leaver et al., unpublished data). This pattern of induction by BNF and PFOA is identical to that observed for the glutathione *S*-transferase gene (GSTA), which contains peroxisomal proliferator response elements (PPREs) and antioxidant response elements (AREs) but not xenobiotic response elements (XREs) in the promoter region (Leaver et al., 1997). Exposure to PCBs generally induces UGT activity in fish species (Table 4.10). When tilapia (*Oreochromis niloticus*) were fed a diet amended with sewage sludge, tissue PCB concentrations were increased, together with an increase in UGT activity (Yang et al., 1993). Coplanar PCBs are AhR ligands; however, when coplanar PCBs are metabolized by CYP isozymes, the hydroxylated metabolites that are formed can be potent inhibitors of UGT and sulfotransferase (van den Hurk et al., 2002).

Inhibition of UGTs

Of great environmental concern is inhibition of the steroid-type UGT by environmental pollutants. Inhibition of these enzymes could cause an accumulation of active hormone that may lead to disturbed gonadal cycles and even tumor formation in the hormone secreting organs. Some xenoestrogens were indeed demonstrated to affect UGTs in fish species; for example, nonylphenol inhibited steroid-conjugating UGT activity in Atlantic salmon (Arukwe et al., 1997), and nonylphenol diethoxylate inhibited UGT activity in rainbow trout hepatocytes (Sturm et al., 2001). Growth hormone caused an inhibition of UGT with testosterone as substrate (Cravedi et al., 1995), indicating that there may be interactions between different UGT isoforms through their respective substrates. In addition, liver UGTs are important in the metabolism and excretion of thyroid hormones in fish; thyroid glucuronides have been measured in the bile of a variety of fish species (George, 1994). In rainbow trout (*Oncorhynchus mykiss*), injection

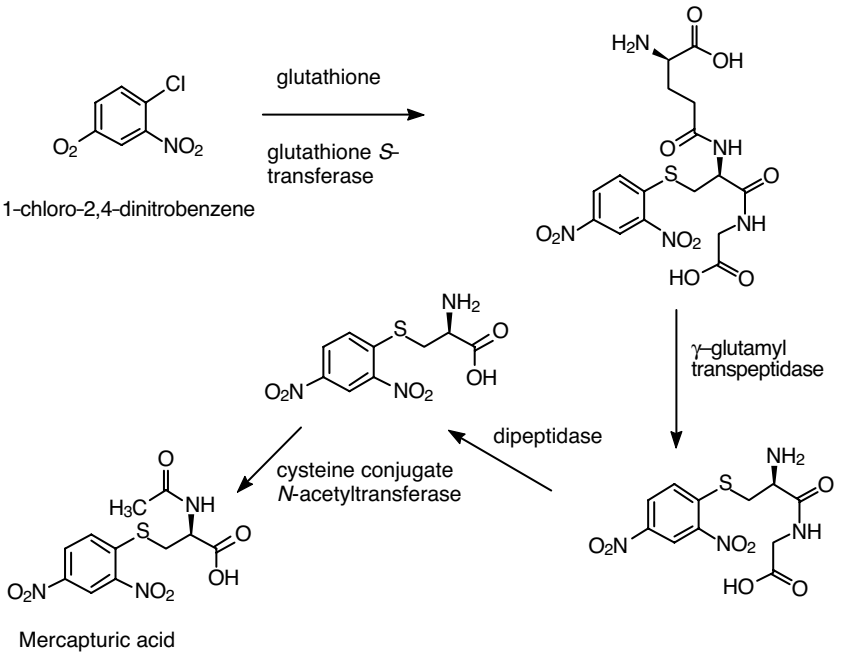


FIGURE 4.11 Glutathione conjugation of 1-chloro-2,4-dinitrobenzene. This compound is a substrate for several distinct glutathione *S*-transferase enzymes and is commonly used to measure glutathione transferase activity. The product has a strong ultraviolet absorbance at 344 nm, and its formation is readily followed spectrophotometrically. Following glutathione conjugation, the conjugate is converted in a three-step reaction to the *N*-acetylcysteine conjugate (mercapturic acid).

of thyroid hormones demonstrated that glucuronidation plays an important role in maintaining a homeostatic thyroid status (Finnson and Eales, 1999). Other environmental toxicants that inhibit UGTs are chlorinated hydrocarbons. Pentachlorophenol and other compounds in pulp mill effluents were shown to inhibit UGT activity in rainbow trout (Castren and Oikari, 1987). Hydroxylated PCBs form another group recognized as potent inhibitors of phenol-type UGT in channel catfish (van den Hurk et al., 2002). Inhibition of phenol-type UGT by PCB metabolites may hinder the detoxification of procarcinogens, as was demonstrated with benzo(*a*)pyrene-7,8-dihydrodiol in channel catfish (James et al., 1994).

Glutathione *S*-Transferases

Overview

The glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a supergene family of phase II enzymes that provide cellular protection against the toxic effects of a variety of endogenous and environmental chemicals. These dimeric enzymes are ubiquitously distributed and comprise approximately 2 to 4% of total cytosolic proteins in liver. The most important reaction catalyzed by all isoforms is the conjugation of the tripeptide glutathione (gamma-glutamyl-cysteinyl-glycine) with an electrophilic center that can be a C, N, or S atom; these are present in arene oxides, aliphatic and aromatic halides, and α,β-unsaturated carbonyls. Following formation of the glutathione conjugate, the metabolite may undergo two separate amino acid cleavage reactions followed by *N*-acetylation to form mercapturic acid derivatives (Figure 4.11). The substrate specificity of the GSTs is extremely broad. Notable toxic xenobiotic electrophilic compounds that are conjugated and of toxicological interest include carcinogens and their metabolites, such as aflatoxin B_1, benzo(*a*)pyrene, 7,12-dimethylbenzanthracene, 5-methylchrysene, and pesticides (e.g., alachlor, atrazine, DDT, lindane, methylparathion). A number of GSTs also catalyze biosynthetic reactions of the leukotrienes and prostaglandins, and others act as organic peroxidases and steroid isomerases. A major role in endogenous metabolism is the detoxification of products of oxidative stress

TABLE 4.11

Immunochemical Analysis of GST Class Occurrence in Aquatic Animals

			Anti-Rat GST Subunits				
	Anti-Plaice-GST		Alpha Class			Mu Class	Pi Class
Species	**A**	**B**	**A1**	**A3**	**A4**	**M1**	**P1**
Catfish			++			+	++++
Cod	++++	±	+	NS	±	++	+++
Flounder	++++	+	–	–	±	–	–
Mullet							
Plaice	++++	++++	+++	–	++++	–	–
Turbot	++++	+++	±	±	+	±	–
Rainbow trout	+	±	–	–	±	–	++++
Sea bass							
Sea trout	+	±	+++	+	+++	+++	++++
Salmon	–	+	–	++	–	++++	–
Mussel							++++
Clam							++++

Source: Adapted from George, S.G., in *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Ostrander, G.K. and Malins, D.C., Eds., CRC Press, Boca Raton, FL, 1994, pp. 37–85.

arising from oxidation of lipids, nucleic acids, and proteins—for example, base propenals such as acrolein from DNA oxidation; cholesterol oxide, fatty acid hydroperoxides, and hydroxynonenals from lipid oxidation; and protein carbonyls from protein oxidation. Some isoforms can also exhibit a covalent binding rather than a catalytic role with some compounds. Reactive metabolites of carcinogens (e.g., PAHs) may bind covalently in a suicide reaction that prevents their reaction with DNA; however, many other neutral or lipophilic compounds that are not substrates, including steroid and thyroid hormones, bile acids, bilirubin, fatty acids, and heme, may bind noncovalently in a reversible manner. Functionally, the significance of this noncovalent binding is unknown, but, considering the high concentration of the enzyme proteins in the cytosol, roles in intracellular transport, as a buffer for these compounds, and as an efflux system (via the ATP-dependent glutathione conjugate efflux pumps GS-X, MOAT, and Mrp) have been postulated.

GST Gene Structure

Glutathione *S*-transferases are widely distributed in nature, and essentially all eukaryotic species contain multigene families, many of which contain further subfamilies of proteins. Both cytosolic and membrane-bound forms are present (Board et al., 1997, 2000; Hayes and Pulford, 1995). They are generally classified according to sequence homology and assigned to seven separate families of cytosolic enzymes (designated class alpha, mu, omega, pi, sigma, theta, and zeta) and to two membrane families—microsomal and mitochondrial (kappa). The native cytosolic enzymes are present as dimers of 24- to 26-kDa subunits, and a characteristic of the different families is that within each family the proteins contain conserved amino acid residues that enable formation of both homo- and heterodimers of enzyme subunits. The microsomal enzymes are trimers of approximately 15-kDa subunits and are integral membrane proteins.

In lower vertebrates and invertebrates, few GSTs have been fully characterized, although on the basis of the broad spectrum of catalytic activities found, immunochemical comparisons, and nucleotide sequence homologies, the presence of multiple isoforms from a number of gene families in all phyla is a certainty. On the basis of immunochemical cross-reactivity with antisera exhibiting family specificity, proteins of the alpha, mu, pi, and theta-like families have been identified in many fish species (Table 4.11), and in three mollusk species pi-class enzymes are the major isoforms.

The relative abundances of these isoforms differ between species. GSTs are most abundant in the liver. The predominant isoform in the cyprinids, salmonids, and gadoids is a pi-class homolog, and the

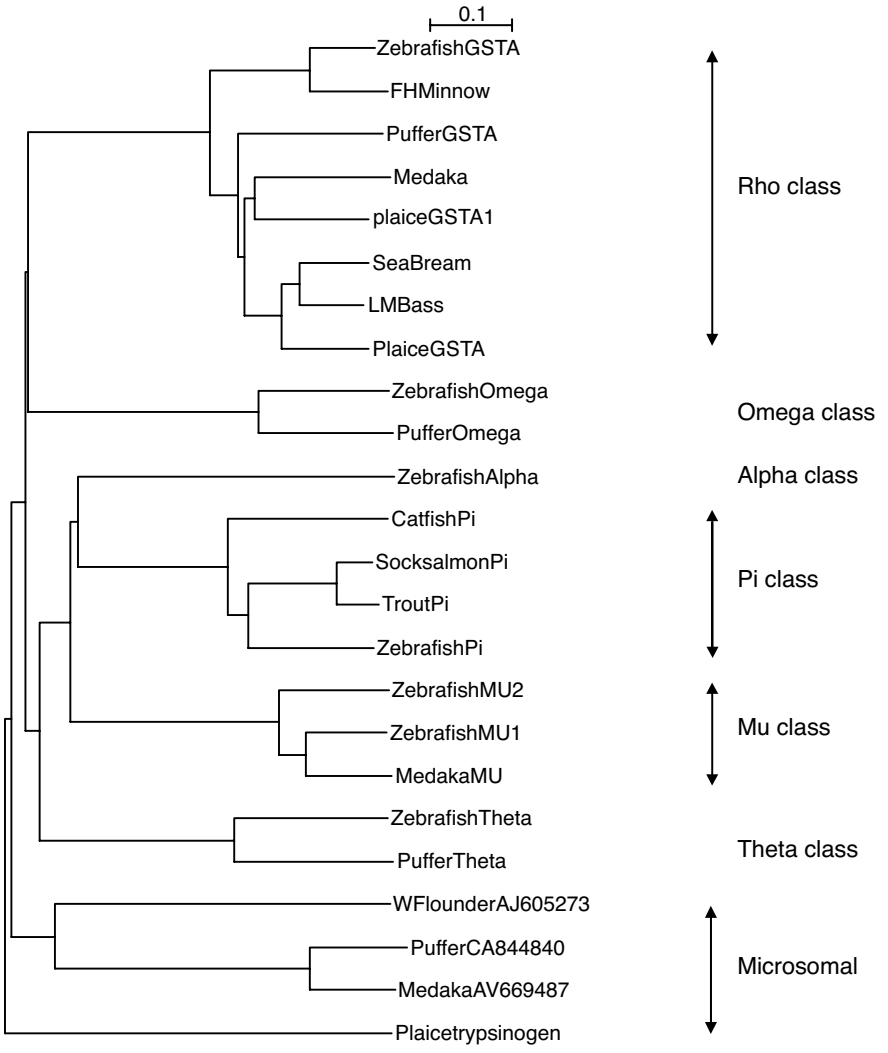


FIGURE 4.12 Phylogenetic relationships of cloned fish GSTs; derived from deduced amino acid sequences using Clustal W (zeta and mitochondrial kappa isoforms omitted).

major isoform in flatfish, mullet, and bass is now designated as Rho-class (Konishi et al., 2005). From nucleotide and protein sequence data, genes for all nine families have been identified in fish to date. A phylogenetic analysis of the sequenced fish genes is shown in Figure 4.12. Table 4.12 shows for each species the number of GST isoforms assigned to each family.

From purification studies, pi-class GSTs have been identified as the major hepatic and intestinal GSTs in the livers of salmonids, brown bullhead, channel catfish, and lamprey. These piscine pi-class enzymes display high activities with BaP epoxides and diol epoxides. The most highly characterized piscine GSTs are from the plaice, a pleuronectid flatfish, where purification and cloning studies identified a multigene cluster of enzymes originally designated as GSTA. These are most closely related to the mammalian, insect, and plant theta-class enzymes and have now been designated as a separate class, Rho. They appear to be unique to fish. Their genetic organization appears to be the same in plaice, flounder, sea bream, and largemouth bass. The primary role of these theta-like enzymes is in oxidative defense. GST-A is most active toward hydroxynonenals, and GSTA1 (or GSTR2) exhibits a high peroxidase activity with organic peroxides. They are the major isoforms in most tissues of flatfish. Homologs have been identified by cDNA sequencing, purification, or immunochemical cross-reactivity in a number of other fish species,

TABLE 4.12

GST Gene Family Homologs Identified by Cloning in Fish Species

Species	Gene Class						
	Alpha	**Mu**	**Omega**	**Pi**	**Rho**	**Theta**	**Microsomal**
Bass	2				2		
Catfish		1		1			
Flounder	1	2			2	1	2
Medaka		1				1	1
Plaice					2		
Pufferfish	1	1	1		2	2	2
Sea bream					1		
Zebrafish	3	3	2	1	1	2	1

Note: Numbers represent quantity of homologs.

and quantitatively they also represent the major isoforms in bass and mullet livers (Gallagher et al., 2000; Martinez-Lara et al., 1997).

Reactions and Substrate Specificity

Before cloning studies and in the early days of purification, attempts were made to identify diagnostic substrates or reactions that may be diagnostic of the presence of certain isoenzymes in different tissues and species. Table 4.13 shows the preferred substrates (apart from 1-chloro-2,4-dinitrobenzene [CDNB]) of the different GST subunits in the rat on which this approach is based. It is important to note that a relatively small number of GST isozymes possess ketosteroid isomerase activity and catalyze the conversion of Δ5-3-ketosteroids to Δ4-3 ketosteroids.

It must be realized that great caution must be exercised when using catalytic activities (CDNB) to evaluate GST expression, as the activity toward any one substrate is a function of both the overlapping substrate specificities of the different isoforms and their different abundances within tissues; thus, such predictions may be unreliable. Consider, for example, the use of activity with ethacrynic acid (ETHA) which is diagnostic of pi-class GSTs but can also be conjugated by an alpha-class enzyme (GSTA4) in

TABLE 4.13

Selected Characteristic Reactions of the Rat GST Gene Families

Class	Subunit	Activities in Addition to CDNB Conjugation
Alpha	rGSTA1	Cholesterol epoxide, aflatoxin B_1 8,9-epoxide; *androsterone 3,17-dione*, prostaglandin H2 isomerase, *carcinogen binding*
	rGSTA3	Aflatoxin B_1 8,9-epoxide; *peroxidase* (cumene hydroperoxide)
	rGSTA4	ETHA, NBC, *4-hydroxynonenal*
	rGSTA5	Aflatoxin B_1 8,9-epoxide, *peroxidase* (cumene hydroperoxide)
Mu	rGSTM1	BSP, *DCNB*, NBC, BaP 4,5-oxide
	rGSTM2	NBC, 4-hydroxy dodecenal (4-OH nonenal), BPDE, *tPBO*, leukotriene A4
	rGSTM3	Very high activity with CDNB
	rGSTM6	4-OH nonenal, NBC, DCNB (testis and brain)
Omega	—	Prostaglandin D synthase
Pi	rGSTP1	DNA hydroperoxides, *acrolein*, BPDE (no ENPP, ETHA, DCNB, CuOOH)
Sigma	rGSTS1	—
Theta	rGSTT1	*ENPP*, BaP-4,5-oxide (no CDNB)
	rGSTT2	ETHA, tPBO, *menaphthyl sulfate*, CuOOH (no CDNB)
Membrane GST	—	*N-Acetylcysteine*, *leukotriene C_4 binding*

Note: Diagnostic activities are italicized. Substrates: BaP, benzo(*a*)pyrene; BPDE, benzo(*a*)pyrene diol epoxide; BSP, bromosulphalein; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; ENPP, 1,2-epoxy-3-(*p*-phenoxy)propane; ETHA, ethacrynic acid; NBC, *p*-nitrobenzyl chloride; tPBO, *trans*-4-phenyl-3-buten-2-one.

three fish species, including cod, plaice, and trout. The relative hepatic activity ratios are 40 for trout, 4 for cod, and 1 for plaice. Although this would correctly indicate the predominance of a pi-class enzyme in trout and not in plaice (despite the presence of a significant amount of a GSTA4 homolog), it does not reflect immunological data showing that the concentration of GST pi in cod liver is very little lower than that of trout. Thus, the usefulness of diagnostic substrates is limited, and this approach is less reliable than immunochemical investigations.

In lower vertebrates, such as elasmobranchs and teleosts, both the covalent and noncovalent binding activities of the GSTs are very much lower as compared to rodents (Foureman et al., 1987; George and Buchanan, 1990). This may be attributed to the lower abundance of a GSTA1 homolog or an evolutionary adaptation of the enzyme in terrestrial vertebrates, as it has been postulated that terrestrial plant phytoalexins are bound by this protein. Teleost species contain stores of polyunsaturated fatty acids that are readily oxidized by free-radical attack, and this may explain the high constitutive levels of isoforms that detoxify lipid peroxidation products such as the alkenals and hydroxynonenals (e.g., GSTA class and GSTA4 homologs) as they will be better protected against xenobiotic-induced oxidative damage. This is particularly relevant in fish such as the cod and plaice where the fat is stored in droplets within the hepatocytes and not in adipose tissue as in the salmonids.

The substrate specificities, primarily with prototypical and endogenous substrates, have been determined with a number of highly purified preparations or recombinant GSTs from several fish species. In common with mammalian GSTs, they show greatest activity with CDNB as the substrate. The alpha-class enzyme from sea bass conjugates the alkenal *trans*-non-2-enal (N2E) at a higher rate than the prototypical xenobiotic substrates, and of these the highest rates were observed with ethacrynic acid (ETHA) and nitrobutyl chloride (NBC) (Angelucci et al., 2000). This is in agreement with an assignment of the sea bass enzyme as a GSTA4 homolog. The pi-class enzymes from salmon, trout, and catfish all exhibit relatively high rates of conjugation of ETHA, again following the pattern observed with a mammalian GST. The catfish enzyme has high activity with (±)-benzo(*a*)pyrene-4,5-oxide and *anti*-benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide as substrates, showing that it is an effective detoxicant of the active carcinogenic metabolite of BaP (Gallagher et al., 1996).

Fish GST and Oxidative Stress

As mentioned above, in addition to their protective activities toward electrophilic chemicals, certain GST isozymes can catalyze the reduction of cellular peroxides to their corresponding alcohols, as well as conjugate endogenous genotoxic unsaturated aldehydes formed during the peroxidation of membrane lipids (Alin et al., 1985; Hubatsch et al., 1998). Accordingly, the GST pathway in some species is an integral component of the cellular antioxidant defense system. Of the reactive intermediates produced during oxidative stress, 4-hydroxynonenal (4HNE) is a particularly reactive α,β-unsaturated aldehyde that is generated during lipid peroxidation as a result of the degradation of ω-6 polyunsaturated fatty acids (Esterbauer et al., 1991). 4HNE production is accelerated during exposure to a variety of prooxidant environmental pollutants (Figure 4.13). Because of its high reactivity, 4HNE rapidly forms covalent adducts with biomolecules containing nucleophilic sites, such as sulfhydryl groups of glutathione, cysteine, lysine, and histidine residues of proteins, and nucleophilic sites of nucleic acids. In rodents and humans, the alpha-class GSTA4 subclass displays uniquely high catalytic activity toward 4HNE and other α,β-unsaturated aldehydes, suggesting that these enzymes may have distinctively evolved as a secondary line of defense against oxidative injury (Hubatsch et al., 1998). As discussed previously, studies with the marine fish plaice (*Pleuronectes platessa*) have revealed the presence of a GST enzyme (GSTR1) that is a relatively efficient catalyst for the conjugation of a series of unsaturated alkenals and hydroxyalkenals, including 4HNE, but displaying little or no activity toward model substrates for mammalian GST. The recombinant Rho class of enzymes in plaice displays higher rates of conjugation of the natural substrates *trans*-oct-2-enal (O2E), N2E, and 4-hydroxy-2,3-*trans*-nonenal (4HNE) than prototypical substrates (apart from CDNB) (Leaver and George, 1998; Martinez-Lara et al., 2002). They also exhibit a high glutathione-dependent peroxidase activity with cumene hydroperoxide; substrate activity with phospholipid hydroperoxides has not been studied. Although both isoforms conjugate 4HNE at the same rate, GSTR1 displays a two- to tenfold higher activity toward O2E and N2E and also shows low but measurable activity with

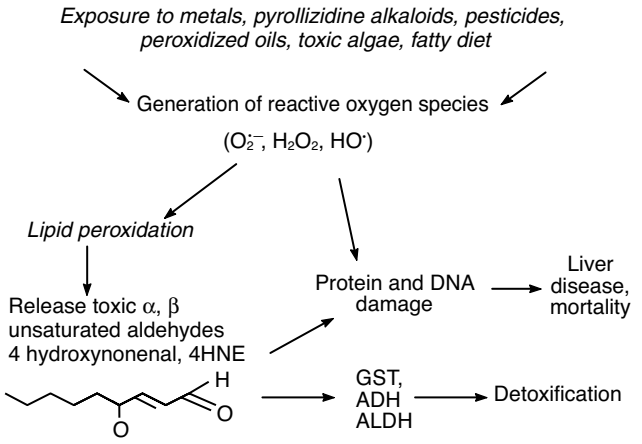


FIGURE 4.13 Pathway for the generation and detoxification of 4-hydroxynonenal (4HNE). In mammals and certain fish species, GST constitutes a protective pathway against 4HNE injury.

the prototypical xenobiotic substrates, with ETHA being the highest. GSTA1 has a fivefold higher peroxidase activity (25 μmol/min/mg protein). The purified GSTA homolog of sea bass displays the same characteristic profile of a high activity with N2E and relatively high activity with ETHA compared with other substrates.

Largemouth bass (*Micropterus salmoides*) expresses a liver GST that shares extensive sequence identity to the aforementioned 4HNE-metabolizing GSTA isolated from plaice that detoxifies 4HNE. Interestingly, the bass GST exhibits a catalytic activity toward 4HNE that exceeds that of several mammalian and aquatic species (Doi et al., 2004; Pham et al., 2002). Bass GSTA is also similar to a GST form found in two other fish species (European flounder and fathead minnow); however, similar genes may not be present in other aquatic species. Bass GSTA exhibits little sequence identity (21% or

TABLE 4.14

The Catalytic Properties of Glutathione *S*-Transferase in Cytosol and Glutathione Affinity-Column-Purified Fractions from Several Fish Species

			Activity (nmol/min/mg protein)		
Species	**Preparation**	**Ref.**	**BPDE**	**EA**	**CDNB**
Catfish (*Ictalurus punctatus*)	Liver cytosol	Gallagher et al. (1996)	5.3	37	1325
	Affinity purified fraction		128	—	24.000
	Intestinal cytosol	Gadagbui and James (2000)	8.3	65	1010
	Affinity purified fraction		453	2880	107,000
Brown bullhead (*Ameiurus nebulosus*)	Liver cytosol	Henson et al. (2001)	<5	—	1130
	Affinity purified fraction		310	—	5,700
Rainbow trout (*Oncorhynchus mykiss*)	Liver cytosol	Melgar Riol et al. (2001)	—	20	630
	Affinity purified fraction		—	2030	77,180
Atlantic salmon (*Salmo salar*)	Liver cytosol	Novoa-Valinas et al. (2002)	—	70	290
	Affinity purified fraction		—	4350	16,600
	Kidney cytosol	Novoa-Valinas et al. (2002)	—	60	200
	Affinity purified fraction		—	10,620	37,500
Brown trout (*Salmo trutta*)	Liver cytosol	Novoa-Valinas et al. (2002)	—	90	650
	Affinity purified fraction		—	3790	27,690

Note: BPDE, (+)-*anti*-benzo(*a*)pyrene-7,8-dihydrodiol-9,10-oxide; EA, ethacrynic acid; CDNB, 1-chloro-2,4-dinitrobenzene.

less) to several mammalian GSTs, including the rat alpha-class GST form rGSTA4-4, which, as discussed, has a high catalytic efficiency toward conjugation of GSH with 4HNE. The presence of this interesting GST in several fish species suggests an important conservation of function that likely protects fish lipid membranes against the deleterious effects of oxidative injury.

Tissue Distribution

With the exception of the mammalian theta-class enzymes, all GSTs conjugate the prototypical substrate 1-chloro-2,4-dinitrobenzene (CDNB) with greatest activity (Figure 4.11). CDNB conjugating activity has been observed in all fish species examined to date (Table 4.14) and occurs in multiple tissues (George, 1994). Because CDNB is a general substrate and the rate of conjugation can vary between isoforms by up to two orders of magnitude, comparison of total CDNB conjugating activity in livers of different species, for example, is probably of little relevance in toxicological evaluation of environmentally relevant xenobiotics, as it will bear little relationship to the rate of metabolism of other compounds which may be isoform specific. Functional comparisons of activity toward individual toxicants is perhaps more meaningful from a toxicological viewpoint. Whereas kidney is the most active toward styrene-7,8-oxide and BaP-4,5-oxide relative to other tissues in the little skate (*Raja erinacea*) (Bend et al., 1978; Foureman et al., 1987; Gill et al., 1982), this finding must be placed in the context of relevance to the animal as compounds of this type may not be present systemically. They will more likely be taken up in the diet or generated by metabolism in the intestine or liver and conjugated in these tissues.

In general, most *in vitro* laboratory investigations of GST–CDNB conjugating activities in fish tissues use assay conditions and substrate concentrations similar to those proposed for mammals: 1 m*M* GSH and 1 m*M* CDNB) (Habig and Jakoby, 1981). Kinetic studies, however, have demonstrated that these substrate concentrations may not be at saturation with respect to initial rate kinetics for many isoforms because the K_m values differ by up to two orders of magnitude. Because the enzymes contain both electrophile and nucleophile binding sites, GST detoxification rates are determined by the concentration of electrophilic substrate and by the concentrations of nucleophilic cosubstrate (GSH). Kinetic studies in largemouth bass and brown bullheads in the presence of variable electrophile concentrations suggest that *in vitro* saturation of hepatic GST–CDNB conjugation occurs at higher electrophile concentrations in brown bullheads than in largemouth bass (Gallagher et al., 2000). Such an observation is consistent with a higher capacity for brown bullheads to detoxify electrophilic GST substrates under conditions of high environmental exposure. These observations are important under the prototypical conditions of environmental chemical exposure when the amount of chemical reaching the liver is relatively low and the rate of *in vivo* clearance is directly proportional to the amount of chemical concentrations *in vivo*. Also, as alluded to earlier, it is important to consider that the rate of GST–CDNB activity may not be reflective of rates of GST conjugation of environmentally relevant GST substrates, such as pesticides or epoxide carcinogens. For example, the rates of hepatic GST–CDNB conjugation by starry flounder and English sole are not correlated with the rates of hepatic GST conjugation of (+)-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BPDE) in those species. Specifically, starry flounder catalyze the rate of CDNB conjugation at initial rates threefold higher than is observed in English sole, whereas GST–BPDE activities are threefold higher in English sole as compared to starry flounder (Gallagher et al., 1998). Thus, specific GST conjugation of environmental chemicals in fish must be quantitated under assay conditions where the conjugation of the chemical can be monitored directly.

Although glutathione conjugation is generally associated with detoxification, small halogenated alkenes, such as dibromoethane, are activated by glutathione conjugation. In mammals, theta-class GSTs are particularly efficient in the conversion of dibromoethane to the carcinogenic sulfonium ion metabolite, as shown in Figure 4.14 (Thier et al., 1996). It was shown that a small fish species, the medaka, was quite susceptible to the development of liver cancer following exposure to ethylenedibromide and that a form of GST in liver was increased in the exposed fish, suggesting the presence of a theta-like GST in the medaka (Hawkins et al., 1998). This has been confirmed with cloning studies. Other fish species, such as the pleuronectid flatfish, bass, and mullet, have been shown to have theta-like GST in their livers (Gallagher et al., 2000; Leaver et al., 1993; Martinez-Lara, 1997). It is tempting to speculate that this may account for the high tumor incidence observed in flatfish from some polluted environments.

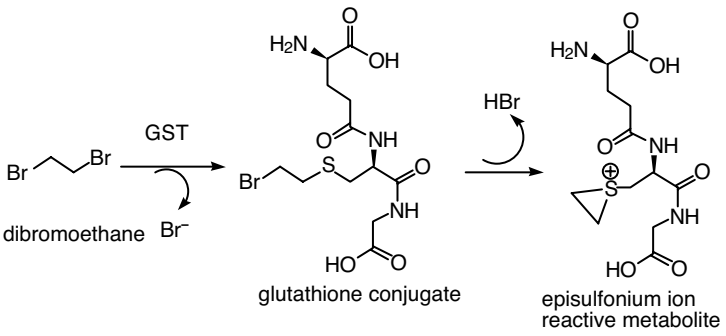


FIGURE 4.14 Formation of a reactive metabolite by glutathione conjugation. Halogenated alkanes such as dibromoethane can undergo glutathione conjugation then eliminate the second halogen to give a reactive episulfonium ion. This electrophilic metabolite can react with cellular nucleophiles.

Regulation of GSTs

Many of the mammalian GST isoforms are inducible; however, GST regulation is complex, and expression is both developmentally regulated and tissue specific (e.g., some are expressed in brain and testis only). Some GSTs are responsive to hormonal effects (growth hormone, thyroxine, insulin), and over 100 xenobiotic compounds have been shown to act as inducers (Hayes and Pulford, 1995). Moreover, in humans and the rat (but not in fish), GSTP1 is overexpressed in hepatocytes during the process of carcinogenesis. From structural and promoter analyses of GST genes (especially rGSTA2), various functional regulatory elements have been identified (Table 4.15). Interestingly, nucleotide sequences contributing to insulin responsiveness of GSTP and to specific expression of GSTs in liver and brain have been identified. Involvement of NF-κB and AP-1 sites, c-*fos*, and c-*jun* are also implicated in regulation. For a detailed discussion, the reader is referred to the review of Hayes and Pulford (1995).

An important facet of the GST pathway from an environmental perspective is the potential for induction of GST isozymes on exposure to certain dietary and environmental chemicals. Typically, a modest induction (twofold or less) of overall GST–CDNB activity is observed in fish exposed to prototypical GST-inducing agents in the laboratory. Studies of GST–CDNB activities in fish may be complicated by variations in diet, water temperature, gender, and reproductive cycling (Swain and Melius, 1984). Furthermore, as discussed, GST–CDNB activity represents an integration of the activity of multiple isoforms, and treatment effects on GST isoforms may not always be distinguishable by analysis of GST–CDNB activity. The importance in ascertaining effects of inducing agents on multiple fish GST isoforms is underscored by the fact that selective modulation of those GST isoforms with high specific activity toward environmental toxicants or their metabolites may be missed if GST–CDNB activity is the only endpoint. Ultimately, modulation of key GST isoforms that primarily contribute to the conjugation of environmental agents (or their metabolites) will be the critical determinants of chemical susceptibility.

TABLE 4.15

Regulatory Elements and Inducer Responsiveness of Rat GST Genes

Element	Inducers
XRE	Planar polyaromatic hydrocarbons, dioxins, etc.
ARE	Phenolic antioxidants, reactive oxygen species, Michael reaction acceptors, quinones, epoxides, etc.
Barbie box element	Barbiturates
GRE	Synthetic glucocorticoids
GPE1 (TRE-related)	Carcinogenesis
Cccgctc	Insulin
HNF1	Liver expression

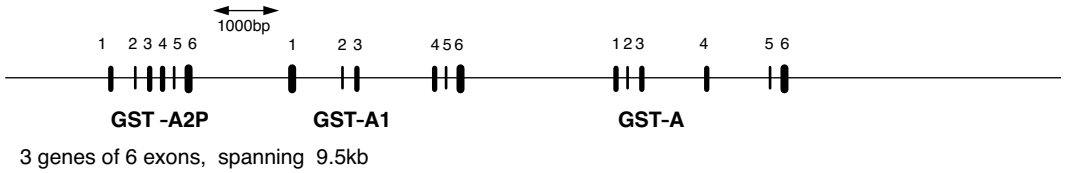


FIGURE 4.15 Structure of the plaice Rho-class gene complex.

Many laboratory studies of GST induction in fish have typically been conducted in conjunction with studies of CYP1A induction and have therefore involved AhR inducers, such as 3-methylcholanthrene (3-MC) and β-naphthoflavone (BNF) (George, 1994). Studies of GST induction in rodents indicate that antioxidant compounds such as ethoxyquin and butylated hydroxyanisole (BHA) are more effective inducers of GST expression than are AhR agonists (Buetler et al., 1995; Hayes and Pulford, 1995). This may not be the case in fish; for example, the level of induction in GST–CDNB activity in bullhead liver (1.6-fold) by ethoxyquin is consistent with previous studies of GST induction by other agents in other fish species. As an example, approximately twofold inductions of GST–CDNB activity have been reported in trout exposed to BNF (Celander et al., 1993) and in plaice (*Pleuronectes platessa*) exposed to BHA or *trans*-stilbene oxide (Leaver et al., 1992). Equivocable results have been obtained in studies on the induction of fish GSTs by xenobiotics, partially due to their high constitutive levels and large inter-animal variations in activities in wild populations, but primarily due to the use of substrates (e.g., HNE, ETHA) that are conjugated by several isoforms or the general substrate CDNB.

Promoter sequences targeted by prototypical GST antioxidant-inducing agents such as BHA not only include antioxidant response elements (AREs) but also electrophile response elements (EpREs), which confer inducibility by the monofunctional phenolic antioxidants to several rat GST and other phase II genes (Li and Jaiswal, 1993; Rushmore et al., 1991). The growing body of studies of GST induction in fish suggests that ARE-mediated GST gene induction may be conserved among many fish species. The GSTA1 gene (Figure 4.15) contains two peroxisomal proliferator response elements (PPREs) that coordinately upregulate expression of both GSTA and GSTA1 after exposure to peroxisomal proliferators (hyperlipidemic drugs, but potentially also environmental contaminants with binding ability such as synthetic lubricants, phthalate ester plasticizers, PCBs, certain pesticides, and alkylphenols). One of these PPREs is associated with an estrogen response element (ERE) that may produce cross-talk and thus explain the sex-dependent differences in expression that are observed. The GSTA gene contains four upstream antioxidant response elements that upregulate expression by compounds which *in vivo* include β-naphthoflavone (or probably a metabolite) and *trans*-stilbene oxide (Leaver et al., 1993). Two of these AREs are associated with CCAAT boxes, which may confer tissue specificity of response (Figure 4.16).

Attempts have been made to utilize GST induction in several invertebrates as biomarkers of xenobiotic exposure; indeed, the pi-class enzymes of Mollusca and Crustacea do show small (one- to twofold) increases in CDNB conjugating activity to many compounds, especially pesticides (Table 4.16). Several studies have also reported increased fish GST activities in fish inhabiting polluted environments (Armknecht et al., 1998; Otto and Moon, 1996). Accordingly, induction of GST activity has been proposed as a biomarker of exposure to environmental pollutants under field conditions. Again, careful characterization of the inducibility of individual fish GST isozymes under laboratory conditions and with respect to physiological status is necessary to interpret field data of GST expression.

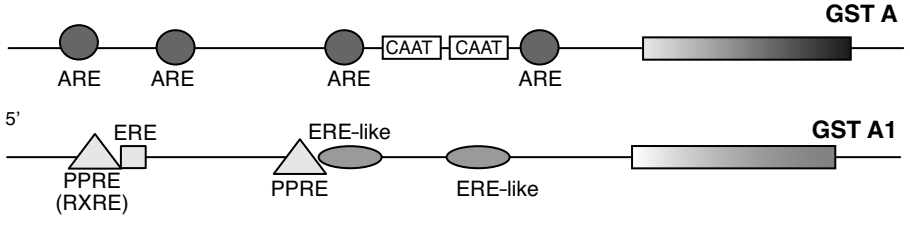


FIGURE 4.16 Promoter regions of the plaice Rho-class genes.

TABLE 4.16

Induction of GSTs by Xenobiotics in Aquatic Organisms

Inducers		Effect
PAHs	3-MC	No effect on activity in minnows and killifish, variable pattern (±1.5-fold in flounder and plaice)
	BNF	No effect on activity, induction of GSTmRNA in plaice, no effect on activity in trout.
	Petroleum/oil	Induced activity in scallop, mussel, and clam
PCBs	Tetrachlorobiphenyl	Induced activity in trout
	Arochlor®	Induced GSTA in flounder and plaice, activity in clams
	Clophen	Slight induction of activity in trout
Reactive epoxide	*trans*-Stilbene oxide	Induced GSTA in plaice and flounder
Antioxidants	BHA	Induced activity in plaice
	Ethoxyquin	Induced activity in salmon and possibly catfish
Pesticides	2,4-D	Induced activity in carp and tilapia
	Azinphosmethyl	Induced activity in carp and tilapia
	Carbaryl	Induced activity in prawns
	Endosulfan, *p,p′*-DDE, methoxychlor	Induced activity in clams
	Cypermethrin	Induced activity in crabs
Peroxisomal proliferators (PPRE-γ agonists)	Clofibrate, perflouroctanoic acid (PFOA)	Induced plaice GSTA and GSTA1
Cyanobacterial toxins	Microcystins	Induced activity in crabs

Inhibition of GSTs

Very little literature exists regarding the inhibition of GST activity; however, compounds that bind covalently (e.g., carcinogens) or noncovalently (e.g., heme degradation products, hematin itself, and bilirubin) will inhibit the activity of isoforms to which they are bound. Some xenobiotics may also be inhibitors of GST. Although the binding constants for bilirubin and hematin for plaice liver cytosolic GSTs are lower than those for mammalian GSTA1, these endobiotics do bind and act as inhibitors with I_{50} values of 320 and 10 m*M*, respectively (George and Buchanan, 1990). Binding constants of these compounds with purified GSTs from elasmobranchs were an order of magnitude lower than with plaice. Of particular note is the very high potency of organotin compounds (tributyltin, triphenyltin) for inhibition of plaice cytosolic GST activity *in vitro* (George and Buchanan, 1990).

Sulfotransferase

Overview

The sulfotransferase (SULT) family of enzymes catalyzes the transfer of the sulfonate group from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to hydroxyl (phenolic or alcoholic) and amine groups in a range of endogenous and exogenous substrates (Coughtrie, 2002; Mulder, 1981):

$$ROH + PAPS \rightarrow ROSO_3H + PAP$$

The cofactor for the sulfation reaction is 3′-phosphoadenosine-5′-phophosulfate and is synthesized from adenosine triphosphate (ATP) and inorganic sulfate. The reaction uses two molecules of ATP per molecule PAPS formed, which indicates the high-energy content of the cofactor (Falany, 1997). The rate of formation of PAPS is relatively slow, and cellular levels of PAPS are usually low. This results in the relatively low effectiveness of sulfation as a biotransformation pathway when organisms are exposed to sudden increase of potential substrates. In addition, the desulfated reaction product of PAPS is still able to bind to the active site of the enzyme and is therefore a potent inhibitor of the catalytic reaction.

Sulfate or sulfamate conjugates, once formed, are acidic molecules with pK_a values in the range of 2 to 4, and they exist largely as anions at physiological pH. Like other anions, they are readily excreted

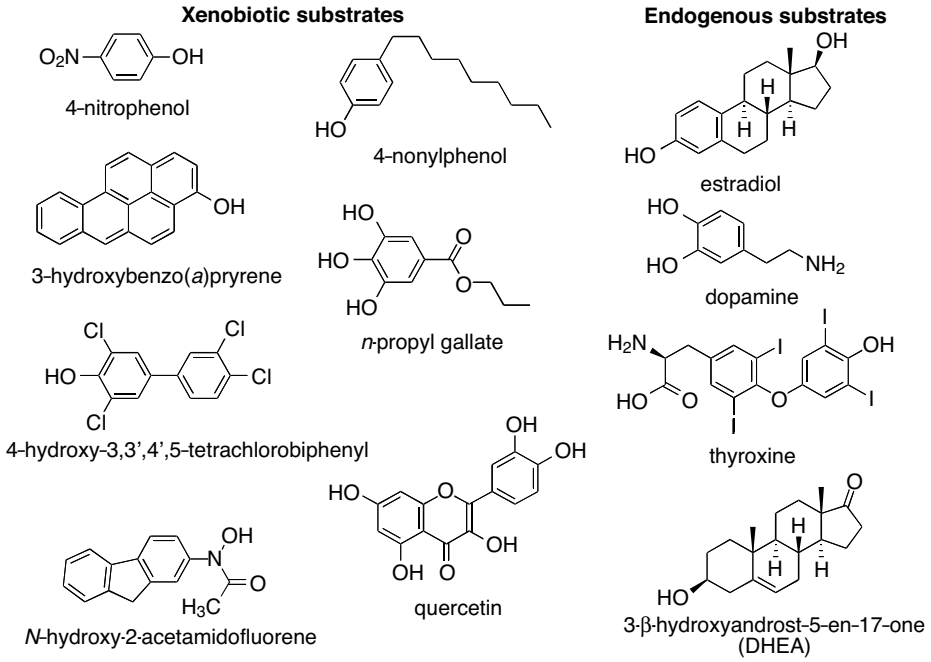


FIGURE 4.17 Selected xenobiotic and endogenous substrates for PAPS–sulfotransferase.

in bile and urine. Sulfate conjugates are also susceptible to hydrolysis by sulfatase, yielding back the parent substrate; for example, sulfate conjugates formed in liver may be excreted in part into the bile. The bile will be secreted into the intestine following a meal, where it may be hydrolyzed back to the starting compound by intestinal sulfatases. The parent compound may be reabsorbed and reconjugated in the liver (where it may undergo another round of biliary cycling) or may be conjugated by glucuronidation or sulfonation in the intestine and excreted.

Pollutant compounds that are potential substrates for SULTs include phenol and phenol derivatives, phase I hydroxylated metabolites of polycyclic aromatic hydrocarbons and polychlorinated biphenyls, aryl amines and their hydroxylated metabolites, and other xenobiotics and their metabolites with hydroxyl or amino groups in the molecular structure. Several physiologically important substrates such as steroids, bile acids, and neurotransmitters are also substrates for sulfation. Examples of xenobiotic and physiologically important substrates are shown in Figure 4.17.

In some cases, sulfonation is a detoxication pathway, but in other cases formation of the sulfate ester or the sulfamate leads to activation of the chemical to a reactive metabolite; for example, sulfonation of hydroxylated (phenolic) metabolites of PAHs is a detoxication pathway (Figure 4.18A). The conjugate is more water soluble and more readily excreted than the parent compound. Similarly, sulfonate conjugates of ring hydroxylated metabolites of *N*-acetylaminofluorene are relatively stable, water-soluble metabolites that are readily excreted. On the other hand, sulfonation of benzylic hydroxy groups, formed from hydroxylation of the methyl group in methylated PAHs (e.g., 6-hydroxymethyl-benzo(*a*)pyrene, 7-hydroxymethyl-12-methyl-benz(*a*,*h*)anthracene), may be an activation pathway (Watabe, 1983) (Figure 4.18B). This is because a sulfate group attached to an aliphatic carbon, especially a benzylic carbon, is a good leaving group and yields the carbocation that can alkylate DNA. In another example, sulfonation of *N*-hydroxyacetylaminofluorene gives an unstable product that decomposes to sulfate and the nitrenium ion, which can alkylate DNA (DeBaun et al., 1968) (Figure 4.19).

In addition to its importance in toxication and detoxication, sulfonation is an important pathway for transport and storage of several endogenous compounds. Estradiol, dehydroepiandrosterone (DHEA), and other hydroxylated steroids are readily sulfonated; they may be transported around the body as the sulfate conjugates and then hydrolyzed back to the active steroid at the target organ (Coughtrie et al.,

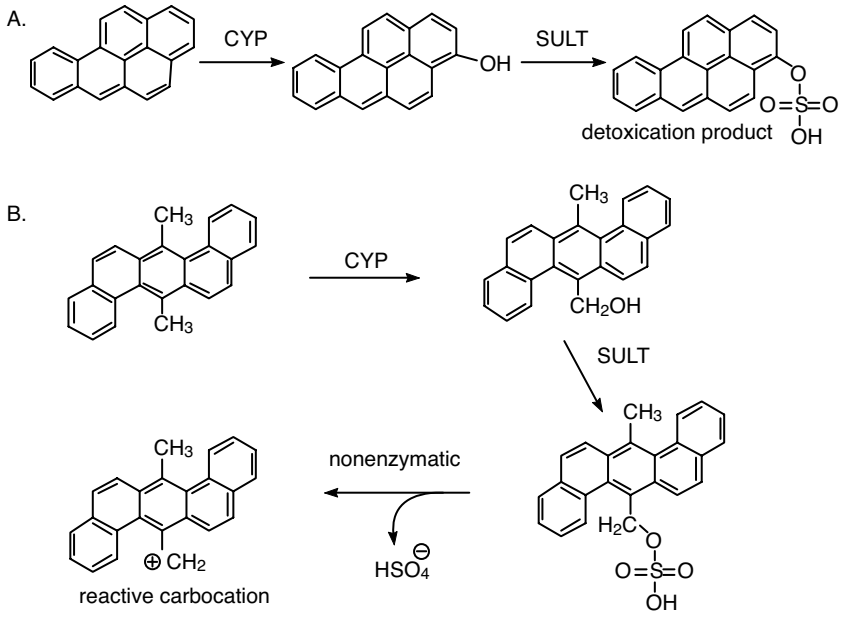


FIGURE 4.18 Sulfonation as a toxication or detoxication pathway. This figure shows examples of polycyclic aromatic hydrocarbons that are metabolized to nontoxic (A) or reactive (B) sulfate esters. The example in (A) shows the oxygenation of benzo(*a*)pyrene to 3-hydroxybenzo(*a*)pyrene, followed by sulfonation of the phenolic hydroxyl group to the nontoxic sulfate ester. The example in (B) shows the oxygenation of one of the methyl groups in 7,12-dimethyl-benz(*a*,*h*)anthracene to give 7-hydroxymethyl-12-methylbenz(*a*,*h*)anthracene, followed by sulfonation of the benzylic (alcohol-like) hydroxyl group to the unstable sulfate ester. This ester can spontaneously eliminate the sulfate group, leaving the reactive carbocation shown. The carbocation will react with cellular nucleophiles, possibly including DNA bases.

1998; Miki et al., 2002). The ability of the sulfonated steroid to cross cell membranes is sometimes superior to that of the unconjugated steroid. This is in part because the conjugates are substrates for several organic anion transporter proteins involved in the uptake as well as efflux of anions and in part because of more favorable solubility characteristics for diffusion conferred by the addition of a polar group to an otherwise poorly water-soluble molecule. The transport and hydrolysis of sulfonate conjugates of steroids have been described in mammals, but to date little direct evidence suggests this pathway in fish. Not only are steroid hormones readily sulfonated but also other physiologically important chemicals such as dopamine, related biogenic amines, and thyroid hormones. In the case of dopamine, the amino group and the ring hydroxyl groups are potential sites of sulfonation. In the shark, the sterol scymnol has been found as the sulfate ester, which appears to serve as a bile acid (Macrides et al., 1997). Rainbow trout liver contains SULT activity that is active with thyroid hormones, especially 3,3′,5-triiodothyronine (T_3) (Finnson and Eales, 1998).

Evidence for the sulfonation pathway in fish has been sought by examining bile, urine, and tank water of exposed fish for sulfonate conjugates, as well as by studying enzyme activity *in vitro* with the substrate of interest (Finnson and Eales, 1996; James, 1986; James et al., 1997, 1998). Studies that demonstrate sulfonation have also been conducted in hepatocytes and with *in situ* isolated preparations (Coldham et al., 1998; Cravedi et al., 1999).

Gene Structure of SULT

Several SULT enzymes with differing substrate selectivities are known to exist in the liver and other organs of humans (Coughtrie, 2002; Dooley, 1998). The nomenclature of the SULT enzymes was originally based on the enzymatic function of the isolated isoforms; however, because recombinant DNA techniques have become mainstream and additional SULT isoforms are being identified by their gene structure, the need for a widely accepted nomenclature was recognized. This has led to a proposed

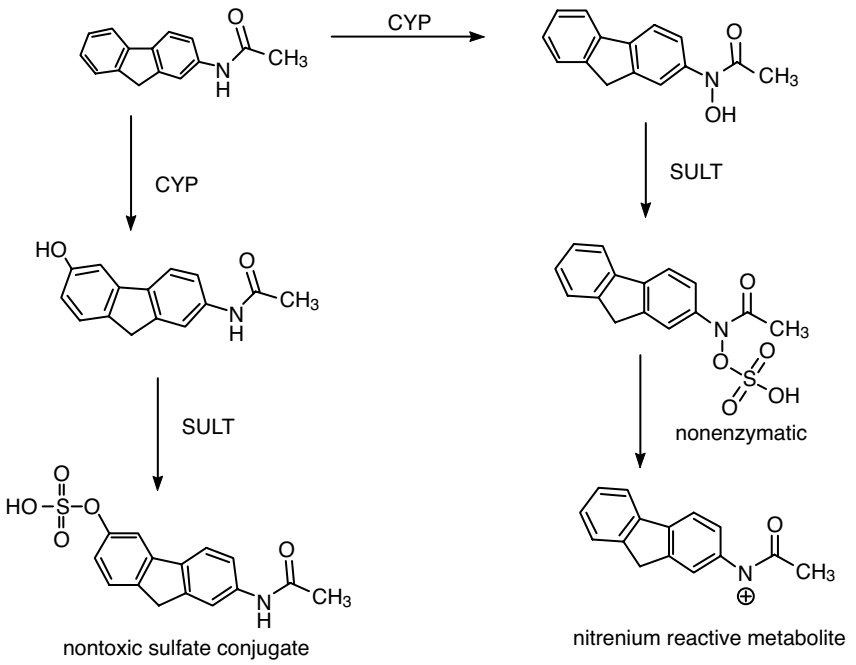


FIGURE 4.19 Sulfonation as a toxication or detoxication pathway. This figure shows two possible pathways of metabolism of the carcinogen *N*-acetamidofluorene. One pathway leads to the nontoxic and readily excreted sulfate conjugate of ring-hydroxylated *N*-acetamidofluorene. The other gives *N*-hydroxy-*N*-acetamidofluorene, which forms an unstable sulfate conjugate that nonenzymatically eliminates sulfate, leaving the reactive nitrenium metabolite. This can bind DNA bases and initiate carcinogenesis.

classification system consisting of a SULT superfamily for all cytosolic SULTs, with families and subfamilies for the individual isoforms being based on their amino acid sequence identity (Blanchard et al., 2004). Two major families comprise the phenol types (SULT1A1), the catecholamine type (SULT1A3), thyroid type (SULT1B), estrogen type (SULT1E1), and DHEA type (SULT2A). Other forms have been identified, but little is known about their biological function.

Recent elucidation of the entire zebrafish genome has allowed searching for DNA sequences that code for SULTs. Using primers based on the PAPS binding site in mammalian SULTs, up to seven different SULTs have been cloned and sequenced from zebrafish (Ohkimoto et al., 2003). Two of these have amino acid sequences that place them in the SULT1 gene family (Sugahara et al., 2003a), and another clone fits into the SULT2 family (Sugahara et al., 2003b). When compared to human and mouse SULT amino acid sequences, there was less than 60% identity, which prohibited classification into existing subfamilies.

The cloned, expressed, and purified zebrafish SULT1 isozymes had a molecular weight of around 35 kDa and demonstrated a strong activity toward 2-naphthol, similar to the mammalian SULTs from this family (Sugahara et al., 2003a). The SULT1 #1 form was more active on dopamine and T_3, while the #2 form was more active toward estrone and thyroxine. When tested for activity toward two coplanar hydroxylated PCBs, the #2 form had a relatively higher catalytic efficiency (V_{max}/K_m). The #2 form loses activity above 45°C, but the #1 form appears to be much more heat stable. Both forms were also analyzed for stability under heavy metal stress. Previous research with mammalian SULT had shown that heavy metal ions can have dramatic effects on SULT activity. The zebrafish SULTs were completely inhibited by 5-m*M* HgCl and CuCl; Co, Zn, Cd, and Pb had a less dramatic but still significant effect (Sugahara et al., 2003a,c). The cloned zebrafish SULT2 isozyme also had a molecular weight of 34 kDa and was active toward DHEA, T_3, and selected neurosteroids but not to any of the other phenolic substrates that were good substrates for the SULT1 isozymes (Sugahara et al., 2003c).

TABLE 4.17

Sulfotransferase Activities in Different Fish Species

Species	Preparation	Substrate	Activity (pmole/min/mg)	Ref.
Guppy (*Poecilia reticulate*)	Liver cytosol	4-Methylumbelliferone	86	James et al. (2001)
		7-Hydroxy-2-acetylaminofluorene	121	
		N-Hydroxy-2-acetylaminofluorene	8	
Medaka (*Oryzias latipes*)	Liver cytosol	4-Methylumbelliferone	130	James et al. (2001)
		3-Hydroxy-2-acetylaminofluorene	25	
Channel catfish (*Ictalurus punctatus*)	Liver cytosol	9-Hydroxy-benzo(*a*)pyrene	600	Tong and James (2000)
	Intestinal cytosol	9-Hydroxy-benzo(*a*)pyrene	930	Tong and James (2000)
		Benzo(*a*)pyrene-7,8-dihydrodiol	2	van den Hurk and James (2000)
Mummichog (*Fundulus heteroclitus*)	Liver cytosol	9-Hydroxy-benzo(*a*)pyrene	8	Gaworecki et al. (2004)
Lamprey (*Petromyzon marinus*)	Liver cytosol	Petromyzonol	232	Venkatachalam et al. (2004)
Rainbow trout (*Oncorhynchus mykiss*)	Liver cytosol	*N*-Hydroxy-2-acetylaminofluorene	0.2	Elmarakby et al. (1995)
	Liver cytosol	Thyroxine (T_4)	7.6	Finnson and Eales (1998)
	Liver cytosol	3,5,3′-triiodothyronine (T_3)	14	Finnson and Eales (1998)

Seven zebrafish SULT isozymes were bacterially expressed and purified and were investigated for activity toward 17β-estradiol and five environmental estrogens. Three of the isozymes did not show any activity, but the other four had various activities toward the substrates (Ohkimoto et al., 2003). The most active isoform (SULT1 ST 2) had a high activity toward 17β-estradiol that was competitively inhibited by bisphenol-A and 4-*n*-nonylphenol. Though the activity toward estrogenic compounds suggests that this isozyme is homologous to the mammalian estrogen form (SULT1E), the amino acid sequence is sufficiently different to exclude it from classification into this subfamily. This zebrafish isozyme also had a high activity toward polyphenolic plant compounds, such as genistein, daidzein, and quercetin. When these phytoestrogens were assayed together with 17β-estradiol, they clearly demonstrated competitive inhibition (Ohkimoto et al., 2004).

Piscine SULTs

Much less is known about these enzymes in fish (Table 4.17). A SULT with activity toward the endogenous substrate scymnol was found in shark liver, but no xenobiotic substrates were studied. The shark SULT had a higher molecular weight (40,000) than mammalian SULT (Macrides et al., 1994). SULT was purified from hepatic and intestinal cytosol of the channel catfish (Falany et al., 1990). Catalytically active fractions from liver and intestine contained proteins of 41,000 MW that cross-reacted with an antibody to the human phenol-sulfating form of phenol sulfotransferase (P-PST). Internal sequence from the liver enzyme indicated it was in the SULT1 family (Tong and James, 2000). The isolated intestinal and hepatic enzymes had nanomolar K_m values with 9-hydroxybenzo(*a*)pyrene as the substrate and were also active with several other substrates.

Reactions and Substrate Specificity

The specificity of sulfonation by substrate has been studied in a few fish species. Sulfonation of *N*-hydroxy-*N*-acetylaminofluorene and 3-hydroxyacetylaminofluorene (procarcinogenic and noncarcinogenic metabolites, respectively of acetamidofluorene) has been studied in small fish used in carcinogenicity testing (see Figure 4.19). As well as the acetamidofluorene metabolites, sulfonation of the model

compound 4-methylumbelliferone was examined. Sulfonation of *N*-hydroxy-*N*-acetamidofluorene was demonstrated in control and 2-acetamidofluorene (AAF)-treated guppy, indicating that the pathway to the reactive ultimate carcinogen was intact (James et al., 2001). SULT activity was higher in guppy than medaka, suggesting a possible biochemical basis for the higher sensitivity of guppy to the carcinogenic effects of AAF. Rainbow trout, a species that is relatively insensitive to the carcinogenicity of AAF, formed very little *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) or N-OH-AAF sulfate (Steward et al., 1995).

Sulfonation of several xenobiotic substrates has been studied in the channel catfish. These include 3,7- and 9-hydroxybenzo(*a*)pyrene, benzo(*a*)pyrene-7,8-dihydrodiol, 2-naphthol, acetaminophen, 6-hydroxymethylbenzo(*a*)pyrene, 7-hydroxymethyl-12-methylbenz(*a*,*h*)anthracene, and *p*-nitrophenol. Higher activity was found with the phenolic substrates than the alcoholic substrates (Tong and James, 2000). In contrast to the benzo(*a*)pyrene phenols, the dihydrodiol procarcinogen BaP-7,8-dihydrodiol was a very poor substrate for sulfonation in the catfish intestine or liver (van den Hurk and James, 2000). Others have shown in mammalian systems that BaP-7,8-dihydrodiol inhibits SULT but is a poor substrate (Rap and Duffel, 1992). *In vivo* studies with the southern flounder, however, have shown that the sulfate conjugate of BaP-7,8-dihydrodiol was formed to some extent and was excreted more rapidly than the glucuronide (Pritchard and Bend, 1984).

The only other fish SULT that has been extensively studied for substrate selectivity is a recently cloned zebrafish enzyme. The expressed zebrafish SULT had high activity with flavonoids, isoflavonoids, and other phenolic natural products such as chlorogenic acid, catechin, epicatechin, and quercetin, as well as with phenolic xenobiotics such as nonylphenol and naphthol (Sugahara et al., 2003a). The zebrafish SULT was also active with dopamine, thyroxine, estrone, and dehydroepiandrosterone. Studies of catalytic efficiency (V_{max}/K_m) showed that the endogenous substrate dopamine was a relatively poor substrate for the expressed SULT enzyme compared with the natural product *n*-propyl gallate (Sugahara et al., 2003a).

Tissue Distribution

Most studies of sulfonation have focused on activity in the liver, but in the catfish the intestine was shown to be as active as liver in catalyzing the sulfonation of several substrates, including hydroxylated benzo(*a*)pyrenes, dopamine, and 6-hydroxymethylbenzo(*a*)pyrene (James et al., 1997; Tong and James, 2000). Demonstration of activity in the intestine is important in the context of first-pass metabolism of dietary carcinogens, which are often present at low levels. When low concentrations of substrate are present in ingested material, biotransformation in the intestine becomes more important. The capacity of the intestine for metabolism is fairly low, because the liver is a larger organ, but if only small amounts of xenobiotic are present, as is the case in many environmental samples, the intestine and other extra-hepatic organs that are portals of entry, such as gills and skin, become important sites of first-pass metabolism.

Regulation of SULT

Not enough studies of SULT in fish have been conducted to have a good understanding of the number of SULTs present or their regulation by endogenous or exogenous factors. In mammalian species, SULTs are not induced by exposure to PAHs, and there are reports that AhR agonists such as PAHS repress SULT activity (Runge-Morris and Wilusz, 1994). The few studies that have been conducted in fish show no clear evidence of an effect of PAH-type inducing agents on SULT activity (James et al., 1997). In catfish exposed to β-naphthoflavone, there was a slight loss of activity with 7-hydroxybenzo(*a*)pyrene but no effect with the 3- and 9-hydroxy-BaP substrates. Evidence suggests that agents such as gluco-corticoids and related substances cause a modest upregulation of SULT in mammals (Runge-Morris, 1998) and that tamoxifen upregulates SULT1A in intestine and liver of rats (Maiti and Chen, 2003), but this has not been demonstrated in fish.

Recent investigations into the expression of phenol-type SULT in mummichog (*Fundulus heteroclitus*) has revealed that this species has a remarkably low expression of this enzyme (Gaworecki et al., 2004). The activity toward 3-hydroxybenzo(*a*)pyrene was very low compared to a variety of other fish species

(Gaworecki et al., unpublished data). In addition, a polyclonal antibody that was generated against channel catfish phenol-type SULT did not recognize any SULT protein in mummichog samples. Despite the low expression of phenol-type SULT in mummichog from clean reference sites, animals from the Atlantic Wood site in the Elizabeth River in Virginia, a site heavily contaminated with PAHs and other contaminants, showed significantly increased SULT activity (Gaworecki et al., 2004). Treatment with single doses of 3-methylcholanthrene or benzo(*a*)pyrene failed to induce the protein; thus, contrary to AhR-mediated CYP1A and UGT induction, SULT upregulation does not appear to be linked to AhR activation. This is not unusual, as GST induction also requires chronic exposure (James et al., 1979).

Inhibition of SULT

Sulfotransferase is subject to inhibition from a variety of sources. *In vitro*, PAP derived from the cosubstrate or sulfonate donor molecule PAPS inhibits SULT activity. Many bulky substrates inhibit their own sulfonation; for example, in catfish intestinal cytosol 3-hydroxybenzo(*a*)pyrene inhibited its own metabolism at concentrations above 0.5 μ*M* (Tong and James, 2000). The nonsubstrate β-naphthoflavone inhibited SULT activity with 3-, 7-, and 9-hydroxybenzo(*a*)pyrene in channel catfish intestinal and hepatic cytosol, with an IC_{50} of 48.0 ± 3.0 μ*M* (James et al., 1997). A natural flavonoid, quercetin, was a potent inhibitor of sulfation of 3-, 7-, and 9-hydroxybenzo(*a*)pyrene in channel catfish intestinal cytosol, with an IC_{50} of less than 1 μ*M* (van den Hurk and James, 2000). Although pentachlorophenol was reported to inhibit phenol sulfation in mammals, it was a poor inhibitor of the sulfonation of hydroxylated benzo(*a*)pyrenes in catfish and had an IC_{50} of about 100 μ*M* (van den Hurk and James, 2000). Recent studies showed that several hydroxylated PCBs inhibited estrogen SULT in mammalian systems with nanomolar IC_{50} values. Studies of the effects of hydroxylated PCB on the sulfonation of hydroxylated benzo(*a*)pyrenes in catfish intestinal cytosol showed that several of these PCB metabolites inhibited SULT, with low to high micromolar IC_{50} values (van den Hurk et al., 2002). Several metal cations, including mercury, cobalt, zinc, cadmium, copper, and lead, were shown to inhibit zebrafish SULT, although quite high concentrations (5 m*M*) were tested (Sugahara et al., 2003c).

Sulfonation of thyroid hormones was studied in liver cytosol from rainbow trout (*Oncorhynchus mykiss*) (Finnson and Eales, 1998). Based on optimum pH, thermal stability, and specific inhibitors, the investigators concluded that it is most likely that only one isozyme form is responsible for the sulfonation of thyroid hormones in trout, in contrast to the multiple isozyme forms found in mammals. No information is available yet on the inhibiting effects of hydroxylated PCBs on thyroid hormone sulfonation, as has been reported for mammalian systems (Schuur et al., 1998).

Activation of SULT

In vitro, SULT activity with some substrates can be increased by the addition of Mg^{2+} and others by incorporation of BSA into the assay vial. This phenomenon was also observed with the isolated catfish SULT (Tong and James, 2000) and zebrafish SULT (Sugahara et al., 2003a). The *in vivo* significance of this activation is unclear (Konishi-Imamura et al., 1995).

Amino Acid Conjugation

Overview

A relatively little studied pathway of phase II metabolism is the conjugation of carboxylic acids with amino acids. This pathway results in the formation of an amide bond between the carboxylate and the amino group of the amino acid (Figure 4.20). The resulting conjugate is an anion at physiological pH and is generally a better substrate for organic anion transporter proteins in kidney than the unconjugated xenobiotic; thus, amino acid conjugation facilitates the excretion of xenobiotic carboxylic acids (James and Pritchard, 1987). The amino acid utilized for this pathway in all fish species studied to date is taurine (James, 1976). It has been suggested that glycine conjugates (the major metabolites of benzoic acids in mammals) are metabolites of *p*-aminobenzoate in fish (James, 1986), but no solid evidence supports this pathway in fish. The enzymes responsible for amino acid conjugation are found in liver and kidney

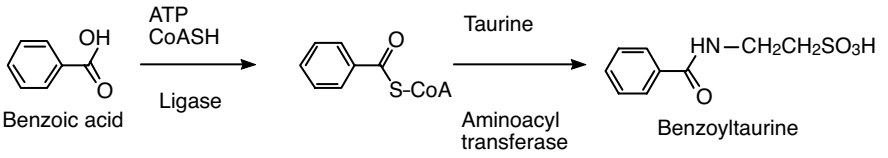


FIGURE 4.20 The pathway of conjugation of carboxylic acids in fish, illustrated with benzoic acid. The benzoic acid forms a coenzyme A thioester, in a reaction catalyzed by acyl–CoA ligase. The benzoyl–coenzyme A thioester then reacts with taurine, catalyzed by aminoacyl transferase, to give benzoyltaurine.

mitochondria. Two enzymes are involved, a carboligase that catalyzes the formation of an acyl-coenzyme A (CoA) intermediate and an aminoacyltransferase that catalyzes formation of the amide bond (Vessey, 1996). Figure 4.20 shows this pathway with benzoic acid as the substrate and benzoyltaurine as the product. Studies of the first step of this reaction—formation of the acyl-coenzyme A—have not been carried out in fish. The second step, catalyzed by acyl-CoA–taurine aminoacyltransferase, is located in the mitochondrial matrix in those fish that have been studied, and kidney had higher activity than liver (James, 1976). The activity has been studied with phenylacetyl-coenzyme A as the substrate and taurine as the cosubstrate. Even when the incubations were supplemented with glycine, no glycine conjugates were found.

Enzymes Specificity, Regulation, and Inhibition

Taurine conjugation is generally considered to be a detoxication pathway. Taurine conjugates are acidic and exist largely in the anionic form at physiological pH. It may be expected that they will therefore be readily excreted in urine. The taurine conjugate of benzoic acid was not, however, as good a substrate for the southern flounder renal organic acid transporter as hippuric acid (benzoylglycine), even though no hippurate was formed in flounder given benzoate (James and Pritchard, 1987). It was, nevertheless, excreted more readily than benzoic acid. Xenobiotic substrates for this pathway include 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), the herbicide triclopyr, and *p*-aminobenzoic acid, a metabolite of the fish anesthetic tricaine methane sulfonate. Endogenous substrates include bile acids, benzoic acid, and phenylacetic acid, the latter being metabolites of aromatic amino acids. Southern flounder have been shown to excrete taurine conjugates of benzoic acid (James and Pritchard, 1987), 2,4-D, and 2,4,5-T (James, unpublished data). Dogfish shark excreted 2,4,5-trichlorophenoxyacetyltaurine in urine following a dose of 2,4,5-T (Guarino et al., 1977). Winter flounder excreted phenylacetyltaurine and 2,4-D-taurine after doses of phenylacetic acid and 2,4-D, respectively (James and Bend, 1976; Pritchard and James, 1979). Channel catfish excreted benzoyltaurine in urine after administration of benzoic acid (Plakas and James, 1990). Triclopyr has not been examined in fish, but a crustacean, the crayfish, retained triclopyr as the taurine conjugate in hepatopancreas and slowly excreted the conjugate (Barron et al., 1991). No studies have been performed on the regulation of this pathway in fish. Inhibitors and activators of the pathway are not known.

Acetylation

Overview

This pathway utilizes acetyl-CoA to donate an acetyl group to an amino group in a xenobiotic. Xenobiotic substrates include arylamines, aliphatic amines, and hydrazines. The reaction pathway with 2-aminofluorene as the substrate is shown in Figure 4.21. The enzymology of *N*-acetylation has been extensively studied in mammals, but few studies of acetylation in fish have been performed, beyond demonstration of the pathway with model substrates (de Wolf et al., 1993; Gregus et al., 1983). Acetylation is also an important terminal step in the formation of mercapturic acid metabolites from glutathione conjugates (Figure 4.11). It is thought, however, that the enzyme that acetylates cysteine conjugates is different from the *N*-acetyltransferase that acetylates xenobiotics containing amino groups that are not part of an amino acid.

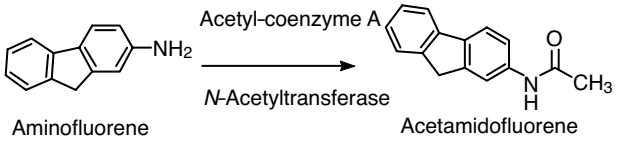


FIGURE 4.21 Acetylation of arylamines. The arylamine aminofluorene forms acetamidofluorene in a reaction catalyzed by *N*-acetyltransferase. The cosubstrate is acetyl–coenzyme A.

Enzyme Specificity, Regulation, and Inhibition

Acetylation has been shown to be a major pathway of metabolism in fish of a number of xenobiotics containing amino groups. These include therapeutic drugs such as sulfadimethoxine (Droy et al., 1989; Kleinow et al., 1992) and pollutants such as aniline, chloroaniline, and quinolines (Birkholz et al., 1989; Bradbury et al., 1993). Acetylation of the procarcinogen 2-aminofluorene leaves the molecule open for further metabolism by *N*-hydroxylation and sulfonation to a reactive nitrenium ion, as described in the section on sulfonation. Another pathway of activation of acetamidofluorene is *N*,*O*-acetyl transfer, leading to the unstable *N*,*O*-acetyl metabolite. This is readily deacetylated to yield a nitrenium ion active metabolite. Thus, although acetylation of amines may improve the water solubility and ease of excretion, it may in some cases lead to the formation of more reactive metabolites. In mammalian species, the *N*-acetyltransferase enzymes are not inducible but are expressed constitutively. The number of forms of *N*-acetyltransferase in fish and their regulation have not been studied.

Toxicological Relevance

The previous sections have addressed the occurrence, regulation, and catalytic activities of biotransformation systems in fish. The following section focuses on three model compounds and chemical classes and the contribution of biotransformation toward the toxicology of each compound. Benzo(*a*)pyrene was selected as being representative of the large class of polynuclear aromatic hydrocarbons (PAHs). Because of the use of the rainbow trout as a model organism for aflatoxin-induced hepatocarcinogenesis, significant work has been carried out characterizing the biotransformation of aflatoxin. Finally, due to the significant potential for exposure to fish, the biotransformation of organophosphates and carbamates is discussed.

Benzo(*a*)pyrene

The metabolic fate of PAHs in fish has been extensively studied because PAHs are common pollutants of the aquatic environment and are carcinogens in fish as well as higher vertebrates (for reviews in fish, see Buhler and Wang-Buhler, 1998; Buhler and Williams, 1989; Stegeman, 1981; Stegeman and Hahn, 1994). Benzo(*a*)pyrene (BaP), the model carcinogenic PAH, has received a great deal of attention and has served as a model to better understand PAH biotransformation. This compound is not in itself carcinogenic but becomes so upon biotransformation to reactive metabolites that form adducts to DNA bases and initiate the process of mutagenesis and carcinogenesis. BaP can also be metabolized to products that are not directly carcinogenic, such as quinones, phenols, and dihydrodiols (Figure 4.3). BaP possesses a structural "bay region" that impairs enzymatic detoxification of specific stereoisomer metabolites; for example, conversion of BaP to the (–)-7,8-dihydrodiol by consecutive reactions catalyzed by CYP1A and epoxide hydrolase (EH), respectively, may lead to an additional CYP1A oxygenation at the 9,10 position, producing the ultimate carcinogen, (+)-*anti*-BaP-7,8-dihydrodiol-9,10-epoxide (Jerina et al., 1984). These reactions are readily catalyzed by CYP1A and EH in fish liver (Buhler and Wang-Buhler, 1998; Buhler and Williams, 1989; Stegeman, 1981; Stegeman and Hahn, 1994). This particular metabolite is recalcitrant to a second EH hydrolytic reaction due to steric hindrance resulting from the "bay region" ring system; thus, the reactive intermediate is free to interact with DNA leading to potential mutagenesis and carcinogenesis.

CYP1A is clearly the predominant enzyme responsible for each oxygenation of BaP, but other CYP isoforms are likely contributors. Studies with recombinant mammalian CYP isoforms indicate metabolic activation of several PAHs by CYP1B1 as well as CYP2C9, CYP2C19, and CYP3A4, albeit at slower rates than CYP1A and CYP1B enzymes (Shimada et al., 1999). CYP1A forms are readily inducible in fish by PAH and related planar polycyclic molecules, and this likely is a major factor in the sensitivity of fish to PAH-type carcinogens. In addition, other phase I enzymes may catalyze oxidation reactions (see above). Inhibition of cytotoxicity with indomethacin within trout cell lines that lack EROD activity suggests that prostaglandin-H synthase may contribute to the activation of BaP (Schirmer et al., 2000). Clearly cooxidative pathways should be explored in more detail, particularly in fish that possess significant quantities of unsaturated fatty acids which are prone to oxidation.

Other metabolites with potential biological activity include quinones, which have been suggested to be derived from initial phenolic metabolites. BaP has been shown to be converted to several quinone metabolites, including the 1,6- and 3,6-quinones derived from the 1-hydroxy and 3-hydroxybenzo(*a*)pyrene precursors (Stegeman, 1981). Although numerous biotransformation studies have identified quinones as BaP metabolites (Stegeman and Hahn, 1994; Willett et al., 2000; Yuan et al., 1997), few have explored the potential effects of these metabolites on cell function. Lemaire et al. (1994) reported that BaP quinones were capable of creating hydroxyl radical in hepatic microsomes of flounder and perch. Oxidative damage in larval turbot was observed following BaP treatment with parallel formation of BaP quinone metabolites.

Although quinones and the (–)-7,8-dihydrodiol can lead to greater toxicity of BaP, it should be noted that the majority of the metabolites of BaP are nontoxic. Examples include phenols (at the 1 and 3 positions) which are primarily conjugated as glucuronides and potentially sulfate derivatives (Buhler and Williams, 1989; Stegeman and Hahn, 1994). Glutathione conjugates presumably of the various arene oxides (4,5, 7,8, and 9,10) have also been reported in fish (Gallagher et al., 1996). The 4,5 and 9,10 dihydrodiols of BaP also do not appear to undergo bioactivation.

Tremendous species differences exist in the bioactivation of BaP in fish. Liver microsomes of channel catfish, which appear less susceptible to PAH-induced liver cancer, produce lower levels of (+)-7,8-expoxide (based on lower concentrations of (–)-7,8-dihydrodiol) than cancer-prone brown bullhead catfish (Pangrekar and Sikka, 1992; Yuan et al., 1997). *In vivo* studies by Willett et al. (2000) confirmed the species differences in (–)-7,8-dihydrodiol formation. However, hepatic EROD activities did not correlate with 7,8-BaP dihydrodiol formation, indicating that another enzyme system may be involved in BaP oxygenation (Willett et al., 2000). Ploch et al. (1998) also reported that, although *in vitro* DNA binding by BaP was directly related to hepatic EROD activity in the two species, *in vivo* DNA adducts were not related to hepatic EROD. These results are consistent with studies in carp, which have significantly higher rates of phase I metabolite formation compared to brown bullhead but are more resistant to PAH-induced cancers (Sikka et al., 1990).

The effects of other pollutants and environmental conditions on other metabolic pathways of PAHs, or all xenobiotics, are largely unknown. Dieldrin significantly enhances the biliary elimination of BaP but does not alter the metabolite profile (Barnhill et al., 2003). Tributyltin significantly inhibits BaP biotransformation in Arctic char (Padros et al., 2003). Rainbow trout acclimated to hypersaline conditions had a higher level of phase I metabolites and a shift from dihydrodiol to phenolic metabolites with 3-hydroxybenzo(*a*)pyrene predominant (Seubert and Kennedy, 2000).

In addition to phase I enzymes, species-dependent differences in phase II pathways may also contribute to differences in BaP-induced toxicities between species. Although significant epidemiological and biochemical evidence exists in humans and rodents (GSTM1), few studies have demonstrated a correlation between adverse effects and species-specific phase II metabolism in fish; for example, hepatic EH and GST activities in channel catfish were higher than in brown bullhead (Gallagher et al., 1996; Willett et al., 2000). When comparing overall biliary metabolite profiles in each species, significant differences were not observed in quantity or profile of conjugated BaP metabolites (Willett et al., 2000). Likewise, English sole from contaminated sites in Puget Sound appear to be more sensitive to liver lesions resulting from PAH exposure than starry flounder, but overall GST-catalyzed conjugation of BPDE is threefold higher in English sole (Gallagher et al., 1998). Although glucuronides represent the highest percentage of conjugated BaP metabolites in fish, few studies have identified specific metabolites or evaluated species differences in hepatic UGTs and whether this pathway contributes to species differences in BaP toxicity.

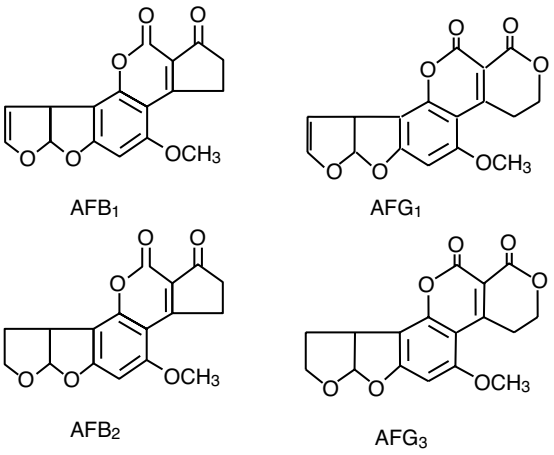


FIGURE 4.22 Structures of naturally occurring aflatoxins.

Although the liver is a major site of biotransformation of BaP, biotransformation to active metabolites in portals of entry, such as the intestine, gills, and skin, is also important. Studies in channel catfish intestine have shown that benzo(*a*)pyrene was metabolized to several metabolites, including BaP-7,8-dihydrodiol-9,10-epoxide, by intestinal microsomes and that metabolism was readily induced by exposure to the AhR agonist β-naphthoflavone (James et al., 1997; Kleinow et al., 1998). The major form of GST present in the intestine was a pi-class GST that had good activity with (+)-*anti*-BaP-7,8-diol-9,10-oxide (Gadagbui and James, 2000). Similar studies carried out with the channel catfish intestinal preparation in which (–)-BaP-7,8-dihydrodiol was infused showed that a major pathway for detoxification of the (–)-BaP-7,8-dihydrodiol was glucuronidation. In the catfish, (–)-BaP-7,8-dihydrodiol was more readily glucuronidated than sulfated, although the rate of glucuronidation of an environmentally relevant concentration of (–)-BaP-dihydrodiol was much lower than the rate of glucuronidation of a similar concentration of BaP phenols; the rate of conjugation of 2.5-μ*M* (–)-BaP-7,8-dihydrodiol in catfish intestinal microsomes was 0.025 ± 0.01 nmol/min/mg protein, whereas that of 1-μ*M* 7-hydroxybenzo(*a*)pyrene was 0.35 ± 0.07 nmol/min/mg protein (James et al., 1997; van den Hurk and James, 2000). In induced catfish, metabolism of (–)-BaP-7,8-dihydrodiol to the ultimate carcinogen (+)-*anti*-BaP-7,8-diol-9,10-oxide was increased, and the capacity of the intestine to detoxify the diol-epoxide by glutathione conjugation was apparently overwhelmed, as induced fish had considerably higher DNA adducts than controls (James et al., 1988). It is likely that species differences in the rate of conjugation of BaP-7,8-dihydrodiol relative to the rate of activation by CYP to BaP-7,8-dihydrodiol-9,10-oxide are factors in the species differences in sensitivity to this carcinogen.

Aflatoxin B_1

The aflatoxins represent a group of closely related difuranocoumarin compounds produced by the common fungal molds *Aspergillus flavus* and *Aspergillus parasiticus* (Figure 4.22). Although fish may be exposed to dietary aflatoxins by ingestion of contaminated feed, especially those with high levels of cottonseed meal, most of the work surrounding the effects of this compound in fish is due largely to its being an excellent model compound for hepatic carcinogenesis in rainbow trout. In the early 1960s, it was discovered that aflatoxin contamination was responsible for an outbreak of hepatocellular carcinomas in hatchery-reared rainbow trout (*Oncorhynchus mykiss*), a species for which background tumor rates are very low (Halver, 1969). Of the various aflatoxins, aflatoxin B_1 (AFB_1) (Figure 4.23) is considered to be the most potent hepatocarcinogen and hepatotoxin (Eaton and Gallagher, 1994). Although the carcinogenic potency of AFB_1 in rats is extremely high, rainbow trout exceed rats in sensitivity to AFB_1 hepatocarcinogenesis and are generally considered to be the most sensitive species to aflatoxin carcinogenicity (Hendricks, 1994). A dietary exposure to 20 ppb AFB_1 of the Shasta strain of rainbow trout for

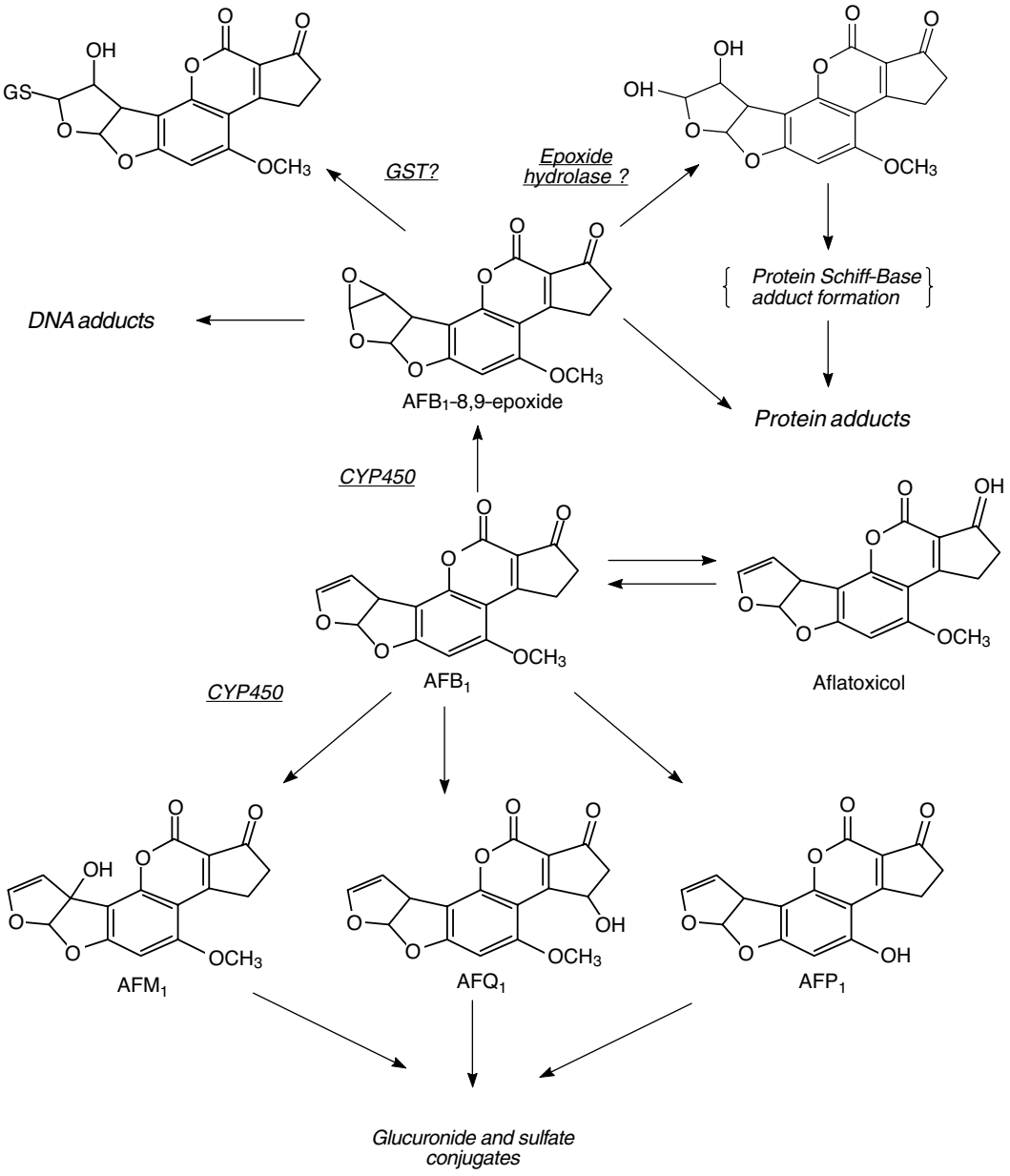


FIGURE 4.23 Biochemical pathways for aflatoxin (AFB_1) biotransformation.

only 4 weeks resulted in a tumor incidence of 62%. Remarkably, static renewal exposure of fertilized rainbow trout eggs (embryos) to 500 ppb (in the bathing medium) AFB_1 for 15 minutes resulted in 62% incidence of hepatic tumors 12 months after hatching (Hendricks, 1994).

Studies in several species have demonstrated that AFB_1 requires microsomal oxidation to the reactive AFB_1-*exo*-8,9-epoxide (AFBO) (Figure 4.23) to exert its hepatocarcinogenic effects. In addition to producing AFBO, chemical and enzymatic epoxidations of AFB_1 can also result in the formation of an *endo*-AFB_1 epoxide stereoisomer (Raney et al., 1992a). Although the *endo*-epoxide is less susceptible to hydrolysis compared to the *exo*-conformation, the *exo*-epoxide is much more efficient at forming DNA adducts and is much more mutagenic than the *endo*-epoxide (Raney et al., 1992b, 1993). AFB_1 carcinogenic potency is highly correlated with the extent of total AFBO–DNA adducts formed *in vivo*

(for a review, see Eaton and Gallagher, 1994). When the administered AFB_1 dose is normalized to target dose (e.g., AFBO–DNA adducts per 10^8 nucleotides), a highly linear relationship between DNA adduct formation and tumor response is obtained, even when using combined data from both rats and rainbow trout (Buss et al., 1990). Furthermore, a large range of linearity exists among total administered dose and AFBO–DNA adduct levels in rainbow trout administered dietary AFB_1 (Dashwood et al., 1988). Collectively, these aforementioned studies demonstrating the linear relationship between AFB_1 dose and AFBO–DNA adducts are not supportive of a threshold hypothesis for aflatoxin genotoxicity at low doses, at least not in two highly sensitive yet diverse species (rats and rainbow trout).

Studies of AFB_1 metabolism and carcinogenesis have demonstrated that AFB_1 biotransformation is intimately linked with its toxic and carcinogenic effects. Accordingly, aquatic species differences among AFB_1 biotransformation pathways are a critical determinant underlying variations in species sensitivities of AFB_1-induced carcinogenesis. Although rainbow trout are extremely sensitive to the hepatocarcinogenic effects of AFB_1, Coho salmon (*Oncorhynchus kisutch*), a closely related salmonid, are resistant to AFB_1 carcinogenicity (Hendricks, 1994). The biochemical basis for this difference in AFB_1 sensitivity among salmonids may be due to less efficient CYP-mediated AFB_1 epoxidation in the Coho salmon relative to the trout. Specifically, AFB_1–DNA binding was reported to be 56-fold greater in rainbow trout liver than in Coho salmon after intraperitoneal (i.p.) AFB_1 administration and 18-fold greater after dietary AFB_1 exposure (Bailey et al., 1988). Other pathways, such as phase II metabolism and AFB_1 elimination, are relatively similar among the two species (Bailey et al., 1988), indicating that microsomal P450-mediated AFB_1 epoxidation and subsequent DNA binding accounts for differences in AFB_1–DNA binding among the two salmonids. Like Coho salmon, microsomes prepared from channel catfish (*Ictalurus punctatus*) liver are inefficient at catalyzing AFB_1 epoxidation (Gallagher and Eaton, 1995). Channel catfish injected with AFB_1 show no elevation in DNA damage as detected by the comet assay, as opposed to rainbow trout, which display extensive DNA damage in blood, liver, or kidney after exposure (Abd-Allah and el-Fayoumi, 1999). Thus, the evidence to date suggests that the resistance of some fish to AFB_1 carcinogenesis can be attributed to inefficient conversion of the procarcinogen to the DNA-reactive metabolite.

The microsomal CYP-dependent monooxygenases also oxidize AFB_1 to its hydroxylated metabolites, AFM_1, AFP_1, and AFQ_1 (Figure 4.23). AFQ_1 is formed via 3α-hydroxylation of AFB_1, whereas AFM_1 is produced by 9α-hydroxylation of AFB_1. *O*-Demethylation of AFB_1 results in the formation of AFP_1. The acute toxicities of the hydroxylated metabolites are generally lower than the parent compound (Hsieh et al., 1974; Stoloff et al., 1972), as are the mutagenic potencies (Coulombe et al., 1982, 1984; Hsieh et al., 1984). However, AFP_1 and AFQ_1 do not appear to be major oxidative metabolites of AFB_1 metabolism in fish. In contrast, significant amounts of AFM_1 are formed in fish (Ramsdell and Eaton, 1990; Ramsdell et al., 1991; Sinnhuber et al., 1974). Dietary AFM_1 is approximately 30% as carcinogenic as AFB_1 in trout (Sinnhuber et al., 1974), whereas the carcinogenic potency of AFQ_1 is approximately 1% that of AFB_1 (Hendricks et al., 1980). Although other aflatoxin metabolites, including the epoxides of AFM_1, AFP_1, and AFQ_1, may contribute to DNA binding, the evidence to date strongly indicates that such secondary oxidation products are of minor importance (Bailey, 1994; Raney et al., 1992b).

CYP2K1 is the major salmonid P450 isozyme that activates AFB_1 to AFBO (Williams and Buhler, 1983; Yang et al., 2000). Immunoquantitation studies of salmonid P450 isozymes indicate that Coho salmon microsomes express less CYP2K1 than rainbow trout, thus providing a mechanistic basis for the lack of AFB_1 oxidation by Coho salmon (Bailey et al., 1988). Juvenile trout injected with the estrogenic and androgen hormones 17β-estradiol and testosterone have lower mRNA and protein levels of CYP2K1 and reduced AFB_1–DNA binding relative to control animals, suggesting that hormonal status may affect the ability of rainbow trout to form the toxic AFBO (Buhler et al., 2000). A CYP2K1 ortholog is also present in zebrafish (*Danio rerio*) (Troxel et al., 1997), and it is likely that this or a related isoform is responsible for the activation of AFB_1 in this species. Like channel catfish, rainbow trout treated with BNF exhibit increased AFM_1 production (Goeger et al., 1988). In general, the capacity for AFBO stereoisomer production has not been measured in fish. It is reasonable to assume that species such as zebrafish and rainbow trout, which can catalyze AFB_1–DNA binding, can form *exo*-AFBO as a significant proportion of their microsomal AFBO. Furthermore, the fact that female zebrafish form more AFBO–DNA adducts than do males may be a reflection of a higher ratio of AFB_1-*exo*/-*endo* epoxide formation for females (Troxel et al., 1997). Interestingly, the level of AFBO–DNA adduct formation in

zebrafish is fourfold lower than that observed for the sensitive rainbow trout but fivefold higher than for the rat. Despite the ability of zebrafish to activate AFB_1 and to form persistent AFBO–DNA adducts, zebrafish appear to be quite resistant to the carcinogenic effects of AFB_1 when administered by the dietary route (Troxel et al., 1997). Thus, the zebrafish studies suggest that mechanisms related to factors other than the inherent ability to bioactivate and detoxify AFB_1 may be involved; for example, it may be that the DNA-adducted cells do not go on to form initiated cells or that a lack of an initiation/promotion progression is present in zebrafish.

As opposed to rodents, fish can rapidly convert AFB_1 to a reductive metabolite, aflatoxicol (AFL) (Figure 4.23), by reducing the 1-keto-moiety via a cytosolic NADPH-dependent reductase (Salhab and Edwards, 1977). Aflatoxicol can be further metabolized by 9α-hydroxylation to form $AFLM_1$ (Figure 4.23) (Lovel et al., 1988). Aflatoxicol is a potent frameshift mutagen and also elicits unscheduled DNA synthesis in fibroblasts incubated with a rat liver postmitochondrial fraction (Stich and Laishes, 1975). Aflatoxicol is approximately 50% as carcinogenic as AFB_1 in trout (Schoenhard et al., 1981) and exhibits about 70% the mutagenicity of AFB_1 in an *in vitro* trout liver activating system (Coulombe et al., 1982). Accordingly, the formation of aflatoxicol does not appear to be an important detoxification pathway for AFB_1, especially as aflatoxicol may be rapidly converted back to AFB_1 by a microsomal dehydrogenase (Salhab and Edwards, 1977), thereby increasing the physiological half-life of AFB_1 (Loveland et al., 1977).

Several of the products of AFB_1 oxidative metabolism serve as substrates for phase II detoxification enzymes. As in the case of AFB_1 epoxidation, there is extensive interspecies variation in phase II conjugation of AFB_1 oxidative metabolites. In mammalian species, the primary pathway for AFB_1 detoxification is through GST-mediated conjugation of AFBO with reduced glutathione (GSH). The selectivity of GST isoenzymes toward AFBO serves as a critical determinant of differences among mammalian species in susceptibility to AFB_1 hepatocarcinogenesis (Degen and Neumann, 1981; Eaton and Gallagher, 1994; Eaton et al., 1990; Lotlikar et al., 1984; O'Brien et al., 1983; Roebuck and Maxuitenko, 1994). Mouse liver cytosolic fractions have 50- to 100-fold greater AFBO conjugating activity than rat even though both species have comparable amounts of GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) (Monroe and Eaton, 1987). Accordingly, mice are resistant to the hepatocarcinogenic effects of AFB_1 when compared to rats (Wogan and Newberne, 1967), a difference reflected by 50- to 100-fold less AFB_1–DNA adduct formation by mice after *in vivo* AFB_1 exposure (Monroe and Eaton, 1987). The high AFBO conjugating activity in mice is due to constitutive expression of an alpha GST isozyme (mGSTA3-3) that has unusually high conjugating activity toward AFBO.

In contrast to mammals, GST-mediated AFB_1 conjugation does not appear to be a significant route of AFB_1 detoxification in fish. Species such as rainbow trout (Valsta et al., 1988), Coho salmon (Valsta et al., 1988), or channel catfish (Gallagher and Eaton, 1995) do not form appreciable amounts of AFBO–GSH conjugates. The low capacity for AFBO–GSH conjugation in the presence of efficient AFB_1 epoxidation in the trout probably accounts for the high covalent binding index for AFB_1 in trout relative to mammals (Bailey et al., 1984). Detectable, albeit low, GST–AFBO activity has been measured in liver cytosolic fractions prepared from English sole (*Pleuronectes vetulus*) and starry flounder (*Platichthys stellatus*) (Gallagher et al., 1998), but the significance of this activity toward AFB_1 sensitivity has not been determined. English sole and starry flounder express a theta-class GST that shows relatively low homology to the mouse alpha-class GST with high AFBO conjugating activity (mGSTA3-3) (Gallagher et al., 1998). A similar form has also been cloned from largemouth bass, although its ability to conjugate AFBO has not been tested (Doi et al., 2004). There is no evidence for the presence of a GST orthologous to the mouse alpha-class GST form that rapidly conjugates AFBO in any fish species examined to date.

As in mammals, glucuronidation is an important pathway for the detoxification and excretion of xenobiotics in fish (for a review, see George, 1994). The production of AFL_1 represents a significant AFB_1 detoxification pathway when a glucuronide is produced and excreted. Biliary AFB_1 conjugates in rainbow trout, zebrafish, and Coho salmon are comprised mainly of AFM_1– and AFL_1–glucuronide conjugates (Loveland et al., 1984; Troxel et al., 1997). A similar metabolic profile appears to exist for channel catfish (Gallagher and Eaton, 1995). Although the rate of glucuronidation of AFB_1 metabolites by catfish liver has not been directly measured, channel catfish have high UDP–GT activities (Ankley and Agosin, 1987; Short et al., 1988) and produce polar biliary AFB_1 metabolites after oral AFB_1 administration (Plakas et al., 1991). Sulfate conjugates were not detected in rainbow trout exposed to

AFB_1 (Loveland et al., 1984), which is consistent with other studies indicating that rainbow trout excrete xenobiotics as glucuronides, as opposed to sulfates (Bailey et al., 1984).

Rodent studies have demonstrated that a variety of dietary factors and synthetic antioxidants have been shown to influence the carcinogenicity of AFB_1. The inducibility of fish hepatic phase II enzymes by synthetic antioxidants has been demonstrated in several species, including plaice (George, 1994), channel catfish (Gallagher et al., 1992), largemouth bass (Hughes and Gallagher, 2004), and brown bullheads (Henson et al., 2001). In the case of the synthetic antioxidant ethoxyquin, juvenile brown bullheads fed a semipurified, antioxidant-free diet supplemented with ethoxyquin exhibited a significant increase in hepatic cytosolic GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) relative to control fish (Henson et al., 2001). Despite the ability of synthetic antioxidants to modulate GST and other phase II enzyme expression, there is little evidence for chemoprotection of AFB_1 carcinogenesis based on modulation of AFB_1 biotransformation in fish. The rainbow trout tumor model has been used extensively to study the mechanisms of a number of dietary anticarcinogens, including AFB_1.

Exposure to the natural product indole-3-carbinol (I3C) was effective as a chemoprotectant against AFB_1 tumorigenesis in rainbow trout (Takahashi et al., 1995). Although it was originally proposed that the mechanism of protection was mediated through the induction of CYP1A activity, studies concluded that the protection was largely due to the fact that I3C undergoes breakdown in the gastrointestinal tract to acid condensation products that act as blocking agents against AFBO–DNA adduct formation (Takahashi et al., 1995).

In summary, AFB_1 is an important dietary carcinogen that can serve as a model substrate for examining aquatic species' differences in biotransformation in relationship to susceptibility to carcinogenesis. The reader is directed to Chapters 5, 6, and 24 for additional information on the roles of biotransformations in the mechanisms of action of environmental procarcinogens in fish.

Organophosphate Esters and Carbamates

Acetylcholinesterases are common targets for a host of xenobiotics, including various classes of pesticides. Biotransformation often plays a critical role in facilitating or impeding the binding of these compounds to cholinesterases. One class of pesticides whose toxicology is significantly affected by biotransformation is the organophosphate ester insecticides. In general, organophosphate esters possess a phosphorothionate group bound to at least two alkyl ethers (see Figure 4.24). The phosphorothionate is usually activated through oxidative desulfuration to an oxon, which irreversibly binds to the serine residues of the anionic site of cholinesterase (see Figure 4.25). The inability of cholinesterase to regenerate because of this covalent interaction results in irreversible binding and inactivation of the enzyme. The classical example is that of parathion, which is activated to paraoxon which inactivates acetylcholinesterase (Figure 4.26). This monooxygenation is classically catalyzed by cytochrome P450 isoforms, but depending on the electronegativity of adjacent functional groups flavin-containing monooxygenases may also be involved in oxon formation (Levi and Hodgson, 1992). Alternatively, various hydrolytic processes (i.e., dearylation of parathion) may also occur which detoxify the compound, as each hydrolytic derivative does not inhibit cholinesterase. Hydrolysis may occur subsequent to or prior to desulfuration and is catalyzed by a host of carboxylesterases (Maxwell, 1992). An additional detoxification reaction involves the conjugation of certain compounds such as methyl parathion and methyl paraoxon with glutathione (Benke et al., 1974).

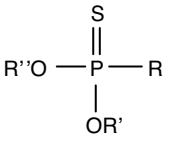


FIGURE 4.24 Structure of organophosphate ester.

Although the toxicity of a host of organophosphates have been examined in fish, the biotransformation of these compounds and elucidation of specific inhibitory metabolites have only been examined in a very few cases. As these organophosphates demonstrate acute toxicity, it is likely that all are converted to oxonic metabolites, even though few have been characterized chromatographically. The most studied organophosphate ester in fish species is parathion. Several studies have reported paraoxon formation in a range of fish species including channel catfish (*Ictalurus punctatus*) (Straus et al., 2000), bluegill sunfish (*Lepomis macrochirus*), bullheads (*Ictalurus melas*), white flounder (*Pseudopleuronectes americanus*) (Hitchcock and Murphy, 1971), sculpin (*Leptacottus armatus*) (Murphy, 1966), rainbow trout (Abbas et al., 1996; Wallace and Dargan, 1987), and mosquitofish (*Gambusia affinis*) (Boone and Chambers, 1997).

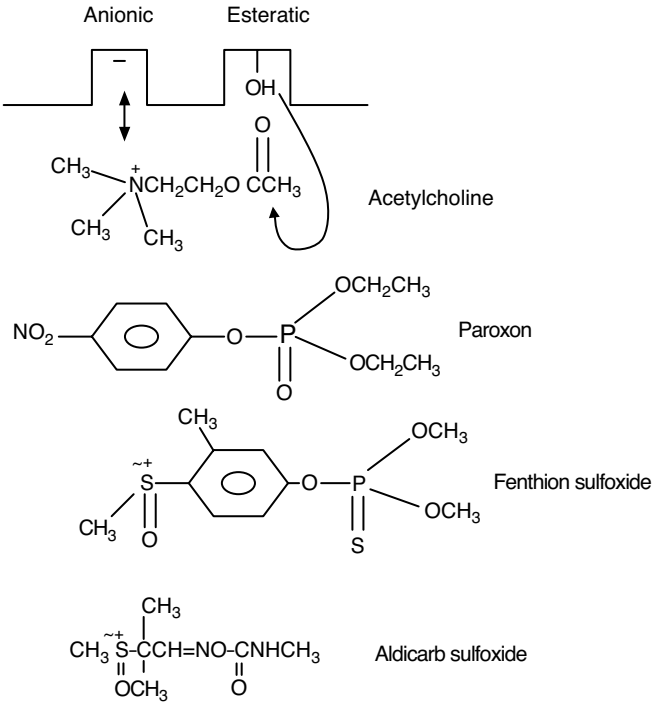


FIGURE 4.25 Inhibition of acetylcholinesterase by aldicarb sulfoxide demonstrating partial positive-charge interactions with enzyme.

Studies in channel catfish have examined the relative differences between dearylation and desulfuration of parathion as well as chlorpyrifos and the role of specific CYP isoforms in these processes (Straus et al., 2000). Pretreatment of fish with Arochlor® 1254 failed to alter either reaction in chlorpyrifos or parathion, indicating CYP1A was not involved in dearylation or desulfuration of either compound. Diazinon is another insecticide in which *in vitro* and *in vivo* biotransformations have been well documented in fish (Keizer et al., 1991, 1993). In fact, differences in oxidative desulfuration have been shown to be responsible for toxicity differences between fish species and developmental stages (Hamm et al., 1998).

Oxidative desulfuration is not the only transformation enhancing the inhibitory potency of cholinesterase-inhibiting xenobiotics. Sulfoxidation of xenobiotics anterior to the phosphorothionate moiety has also been shown to enhance the binding of the xenobiotic to the cationic site of cholinesterase (Figure 4.25). Recent studies in goldfish have shown that fenthion undergoes transformation to the sulfoxide and separately desulfuration to the oxon *in vivo* (Kitamura et al., 2000). *In vitro* studies using hepatopancreas microsomes from goldfish demonstrated conversion of fenthion to the sulfoxide, which was inhibited by SKF-525A and partially by alpha-naphthylthiourea, thus suggesting roles of CYP and FMO in the transformation (Kitamura et al., 1999). Studies with hepatopancreas cytosol indicated reduction of the sulfoxide to the sulfide through aldehyde oxidase (Kitamura et al., 1999). Fenthion sulfoxide was also observed in bioaccumulation studies using medaka treated with fenthion, but neither the sulfone nor oxon was observed (Tsuda et al., 1996).

Although aldicarb is a carbamate insecticide, oxygenation of the sulfur anterior to the carbamyl moiety significantly elevates the toxicity of this compound 40 to 150 times depending on the species (El-Alfy and Schlenk, 2002; El-Alfy et al., 2001; Perkins et al., 1999). *S*-Oxygenation of aldicarb has been observed in rainbow trout, Japanese medaka (*Oryzias latipes*), hybrid striped bass (*Morone saxatilis* × *chrysops*), channel catfish (*Ictalurus punctatus*), and rainbow trout (*Oncorhynchus mykiss*) (El-Alfy and Schlenk, 1998; Perkins and Schlenk, 2000; Perkins et al., 1999; Schlenk and Buhler, 1991; Wang et al., 2001). In each species except catfish, FMOs contributed to the *S*-oxygenation. As channel catfish and other predominantly freshwater fish do not have active FMOs (Schlenk et al., 1993), cytochrome P450

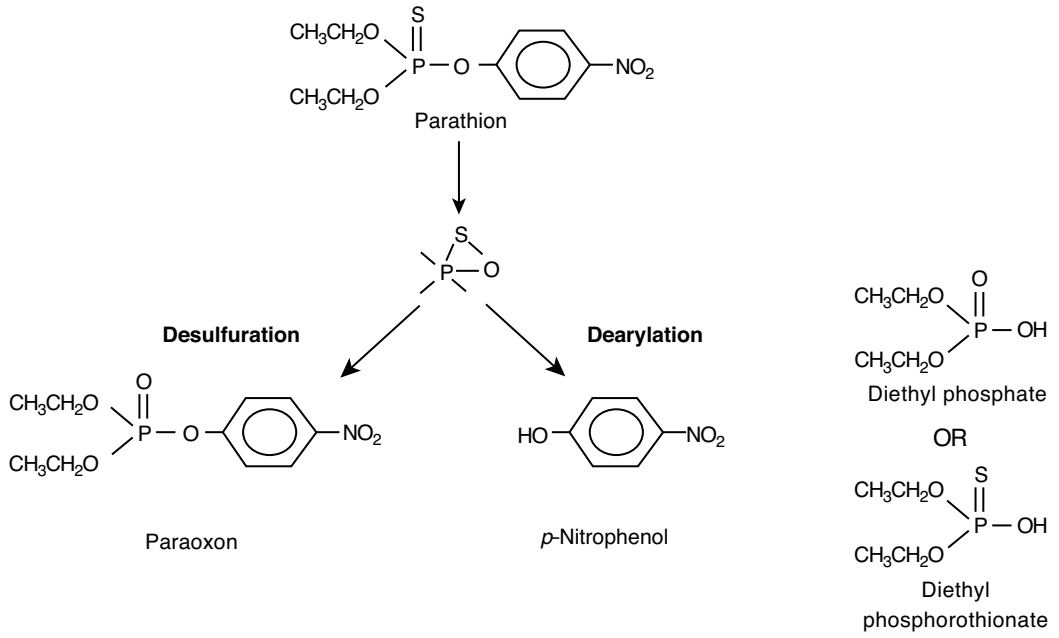


FIGURE 4.26 Metabolic pathway of parathion in channel catfish. (From Straus, D.L. et al., *Aquat. Toxicol.*, 50, 141, 2000. With permission.)

is exclusively responsible for this reaction (Perkins et al., 1999); however, no single CYP isoform appears to predominate in the sulfoxidation of aldicarb. Rather, it is likely that several isoforms participate in the reaction (Perkins et al., 1999). The reduced *S*-oxygenation of aldicarb in channel catfish relative to rainbow trout may explain its resistance against aldicarb toxicity compared to rainbow trout, which readily convert aldicarb to aldicarb sulfoxide (Perkins and Schlenk, 2000).

In summary, fish are capable of activating organophosphate esters to more potent cholinesterase inhibitors. Species differences in the bioactivation and esterase-mediated cleavage of organophosphates have been shown to greatly contribute to species differences in toxicity to these compounds. These data argue for more studies to better characterize specific enzymes responsible for these transformations to help identify sensitive populations of species that may be severely impacted by these compounds.

Conclusions

Biotransformation can be a very important process in the disposition and mechanistic determinations of the mode of action of xenobiotics. Alterations in enzyme expression can dramatically affect the sensitivity of an organism to the toxic insult of a xenobiotic or the disposition of endogenous substrates. Alteration may occur as a result of genetics, diet, gender, environmental influences, or other xenobiotics. Understanding the latter is critical in risk evaluations of chemical mixtures. Very little is known regarding the substrate specificities or the regulation of biotransformation enzymes in fish. Through advances in genomic technologies and the use of fish models in human health research (e.g., zebrafish, medaka), numerous genotypic discoveries have recently occurred. It is likely that phenotypic functionality studies with heterologously expressed enzymes resulting from genomic examinations will help in better understanding biotransformation pathways in fish. In addition, more studies are needed with whole-animal systems to better characterize *in vivo* pathways of chemical biotransformation in species other than classic fish models. Such studies are imperative for physiologically based toxicokinetic (PBTK) models, which may help better estimate dose and aid regulators in reducing uncertainty between species, thus leading to more accurate evaluations of chemical risk.

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5

Receptor-Mediated Mechanisms of Toxicity

Mark E. Hahn and Eli V. Hestermann

CONTENTS

Introduction

Chemicals cause toxicity by interfering with the structure and function of cellular macromolecules such as proteins (e.g., enzyme inhibition and inactivation), lipids (e.g., lipid peroxidation), and nucleic acids (e.g., formation of DNA adducts). Chemicals or their metabolites also may act through interference with physiological signaling pathways, often by altering gene expression. In some cases, biotransformation of the original chemical modifies toxicity, either enhancing it through bioactivation to more reactive derivatives or reducing it through inactivation and enhanced excretion.

This general description of mechanisms of toxicity is as true for fish as it is for humans and other animals. In fact, most mechanisms of toxicity are not specific to fish. Thus, to understand mechanisms of toxicity in fish it is necessary first to obtain a strong foundation in mammalian toxicology, where

much of the research and many of the major advances in mechanistic understanding have occurred. A discussion of mechanistic toxicology in fish is in part a question of extrapolation of knowledge obtained in rodent and human systems to fish. How do biochemical systems and mechanisms of toxicity in fish compare to what has been established in mammals? Are there differences in diversity or function of receptors and biotransformation enzymes involved in these mechanisms? Fish also have many unique features as compared to mammals, including distinct tissues (gill), routes of exposure (aqueous), and life history (externally developing eggs, metamorphosis). How do these influence the ability to extrapolate from rodents, or provide opportunities for novel mechanisms to be discovered? In addition, how can fish be used to study mechanisms involved in the effects of long-term exposure in natural setting—effects such as environmental carcinogenicity or evolved chemical resistance?

This chapter builds on a foundation in mammalian mechanistic toxicology (Gregus and Klaassen, 2001), as well as previous general descriptions of mechanistic toxicology in aquatic systems (Di Giulio et al., 1996; Mommsen and Moon, 2005; Schlenk and Benson, 2001) and other chapters in this book that deal with mechanism-related topics such as biotransformation (Chapter 4), oxidative stress (Chapter 6), target-organ toxicity (Chapter 11), and biomarkers (Chapter 16). We focus on mechanisms involving receptors and other ligand-activated transcription factors that regulate gene expression in response to chemical exposure.

Receptors are important in mechanisms of toxicity for several reasons: (1) Numerous receptors have been identified as targets of chemicals or are potential targets based on known ligands (Table 5.1). Chemicals can act as receptor agonists, partial agonists, or antagonists (defined below). Although toxicants may "mimic" the natural ligands in activating the receptor, they may do so at an inappropriate time, in an inappropriate cell type, or in a sustained way rather than the transient stimulus that characterizes most physiological ligands. (2) Receptors are involved in highly coordinated signaling pathways with key roles in embryonic development or adult homeostasis; thus, perturbation of receptor function can have serious and wide-ranging effects on the organism. (3) Receptors often can be activated or inhibited by very low concentrations of chemicals (nanomolar to micromolar) and thus can be sensitive to trace amounts of chemicals in the environment. (4) The effects of receptor activation often include amplification of the stimulus; for example, receptors that act as transcription factors typically control the expression of several genes, and a single activated receptor can stimulate the synthesis of multiple transcript copies from each gene. Other receptors control ion channels or initiate kinase cascades that similarly involve signal amplification.

To illustrate some general principles of receptor-mediated toxicity as they apply to fish, we concentrate on the well-known mechanisms involving dioxin-like compounds acting through aryl hydrocarbon receptors (AhRs); where appropriate, we also discuss other receptors that are targets for environmental contaminants. Important themes in this chapter include the value of fish both as targets and models, ligand–receptor pharmacology and its relevance to mechanisms of toxicity, and the presence in fish of additional diversity of toxicologically relevant genes—the result of a fish-specific whole-genome duplication in a teleost ancestor.

Fish as Targets and Models

The study of mechanisms of toxicity in fish involves two distinct rationales, with fish serving both as targets and as models. Clearly, fish are *targets* for environmental contaminants; some fish populations are highly exposed to chemicals, especially in urban, industrial, or coastal sites. These fish are studied because of concern for population-level effects (Cook et al., 2003; Nacci et al., 2002) or as sentinels for the health of the environment. In addition, they may be subjects of research to investigate the mechanisms involved in physiological or evolutionary adaptation to chemical exposure (Wirgin and Waldman, 2004) (see Chapter 13). Fish also are widely (and increasingly) used as animal *models* in toxicological research, with the ultimate goal of extrapolating the results to inform questions concerning the potential human health effects of chemical exposure. Thus, research in fish toxicology involves a two-way extrapolation, with human (and rodent) results being used to establish mechanisms of relevance to fish as targets, and fish being used as models to address specific mechanistic questions with application to human health.

Several features of fish make them valuable as models in toxicology (Ballatori and Villalobos, 2002; Hinton et al., 2005; Kelly et al., 1998). As vertebrates, fish have a close evolutionary relationship to humans, with shared genes and biochemical pathways that have become even more apparent as a result of recent whole-genome analyses (Aparicio et al., 2002; Jaillon et al., 2004). Most of the fish species used in toxicological research are small, develop rapidly with a short generation time, and have transparent, externally developing embryos that facilitate experiments in developmental toxicology. For some species, such as zebrafish, methods for transgenesis (Linney and Udvadia, 2004; Udvadia and Linney, 2003) and gene knock-down (Nasevicius et al., 2000) are well developed and serve as powerful tools for mechanistic research. These and other advantages of small fish models have been described in detail elsewhere for zebrafish (*Danio rerio*) (Carvan et al., 2005; Hill et al., 2005; Linney et al., 2004b), medaka (*Oryzias latipes*) (Oxendine et al., 2006), Atlantic killifish (*Fundulus heteroclitus*) (Burnett et al., 2007), and rainbow trout (*Oncorhynchus mykiss*) (Bailey et al., 1996).

Comparative Toxicology: Complications and Opportunities

The use of fish as models for human health requires careful consideration of the similarities and differences between fish and mammals. First, it is important to keep in mind that "fish" as a group encompasses an estimated 29,000 species (compared to approximately 5500 for mammals), including bony, cartilaginous, and jawless fishes and representing an enormous range of genetic, biochemical, and physiological diversity. Second, although as vertebrates fish share with humans and other mammals most features of key biochemical pathways involved in mechanisms of toxicity, important differences between fish and mammals in the details of these pathways have emerged in recent years (Table 5.1). Most notable is the finding that a whole-genome duplication occurred in the teleost fish lineage approximately 350 million years ago (MYA), after its divergence from the lineage that would become tetrapods (including humans) (Amores et al., 1998; Christoffels et al., 2004; Crow et al., 2006; Hoegg et al., 2004; Postlethwait et al., 2004; Taylor et al., 2001). The result of this is that many fish species have retained extra copies (paralogs*) of some genes as compared to humans. Although only approximately 15 to 30% of the duplicated genes have been retained (Brunet et al., 2006), many of these encode transcription factors (Brunet et al., 2006; Steinke et al., 2006). Examples of toxicologically relevant genes for which fish have additional paralogs include CYP19 (Kishida and Callard, 2001; Tchoudakova and Callard, 1998), estrogen receptors (ERs) (Bardet et al., 2002; Hawkins et al., 2000), and aryl hydrocarbon receptors (see below). Other differences in genetic diversity have arisen from independent expansion of some gene families in mammals and fishes (e.g., the CYP2 family) as well as from lineage-specific gene losses in these groups. The resulting differences in the toxicological toolkit raise important questions about the extrapolation of findings between fish and mammals. The existence of differences in receptor diversity among species highlights the need to understand the nature of homologous relationships among these genes; interpretations and extrapolation will depend on whether the genes under consideration are related as orthologs or paralogs (Sanetra et al., 2005).

The presence of extra copies of fish genes as a result of the fish-specific whole-genome duplication is more than just an annoying complication. It also provides an opportunity for mechanistic insights, because duplicated fish genes might be exploited to obtain new information about the function of their single mammalian counterpart (Amores et al., 1998; Force et al., 1999; Lynch and Force, 2000; Postlethwait et al., 2004; Taylor et al., 2001); for example, the duplication, degeneration, complementation (DDC) model of gene evolution (Force et al., 1999; Lynch and Force, 2000) predicts that the multiple functions of a mammalian gene may be partitioned between its fish "co-orthologs" (referred to as *subfunction partitioning*) (Postlethwait et al., 2004). Most of the evidence in support of this model comes

* Distinct types of homologous genes occur within and among species, and specialized terms are used to distinguish among them. Genes in two different species are *orthologous* (Fitch, 1970) if they are descended from the same gene in the most recent common ancestor of the two species (i.e., the two genes are separated only by a speciation event). *Paralogous* genes (Fitch, 1970) are homologs that resulted from a gene duplication event such as a tandem duplication or a whole-genome duplication. If a gene duplication has occurred in one lineage but not another, the term *co-ortholog* (Gates et al., 1999; Taylor et al., 2001) is used to describe the relationship between each of the two duplicated genes in one species and their single ortholog in the other species. Other types of homology have also been described (Meyer and Mindell, 2001).

TABLE 5.1

Receptors and Other Ligand-Activated Transcription Factors Potentially Involved in Mechanisms of Toxicity: Diversity in Fish vs. Mammals

Transcription Factor	Dimerization Partner	Examples of Physiological Ligands or Activators[a]	Examples of Xenobiotic Ligands or Activators[b]	Presence and Diversity		Genes Regulated
				Mammals	Fishes	
AhR	ARNT1 (ARNT2 *in vitro*)	(Bilirubin, biliverdin, tryptophan metabolites, fatty acids)	Dioxins, non-*ortho* PCBs, some PAHs, etc.	1 (AhR)	1–6 (AhR1, AhR2, AhR3)	CYP1A, CYP1B, GST, UGT, NQO
AhRR	ARNT	None	None	1	1–2	CYP1A
CAR	RXR	Androstanol/androstenol (inhibits), lithocholic acid, bilirubin	Phenobarbital (PB), TCPOBOP, chlorinated pesticides, *ortho*-PCBs	1	Not found	CYP2B, CYP3A, GST, ABC transporters
PXR (SXR)	RXR	Pregnenalone, corticosterone, bile acids (lithocholic acid)	PB, PCN, *ortho*-PCBs, organochlorine pesticides, dexamethasone, phthalic acid, nonylphenol	1	1	CYP3A, CYP2B, CYP7A, GST, ABC transporters, AhR
PPAR	RXR	Polyunsaturated fatty acids	Fibrate drugs, phthalate esters	3	4–7	CYP4A, CYP7A, CYP8B, LXR
LXR	RXR	Cholesterol, 24(S)-hydroxycholesterol	GW3965, T0901317	2	1	CYP7A, ABC transporters, LXR
FXR	RXR	Lanosterol, bile acids, chenodeoxycholic acid	GW4064, 6-α-ethyl-chenodeoxycholic acid	2 (FXRα, FXRβ)	2	CYP7A, CYP8B, CYP27A, ABC transporters
ER	ER	17β-Estradiol	Structurally diverse xenoestrogens such as *o,p′*-DDE, *p*-nonylphenol	2 (ERα, ERβ)	3 (ERα, ERβa, ERβb)	CYP19, Vtg
ERR	ERR	Unknown	Diethylstilbestrol, tamoxifen, bisphenol A	3	4–6	Acyl-coenzyme A dehydrogenase, osteopontin
AR	AR	11-Ketotestosterone, dihydrotestosterone	Linuron, *p,p′*-DDE	1	2	—
GR	GR	Cortisol	Dexamethasone, ketoconazole (antagonist)	1	2	TAT, CAR, PXR, RXR
RAR	RXR	all-*trans*-Retinoic acid, 9-*cis*-retinoic acid	—	3	4	—
RXR	Various	9-*cis*-Retinoic acid	Methoprene	3	4	—
MTF	—	Zn^{2+}	Cd^{2+}	1	1	Metallothioneins
NRF[c]	Small Maf proteins	Endogenous electrophiles and ROS	Activated by electrophiles, quinones	3 (NRF1, NRF2, NRF3)	3–5	GST, GCL, NQO

[a] Compounds in parentheses indicate natural ligands of possible, but uncertain, physiological relevance.

[b] Xenobiotic ligands or activators as determined in a variety of systems: mammals and/or fish.

[c] NRFs are also known as NFE2-like (NFE2L1, NFE2L2, NFE2L3).

Source: Data sources and additional details can be found elsewhere (Baker, 2005; Chiang, 2003; Gregus and Klaassen, 2001; Hahn et al., 2005; Maglich et al., 2003).

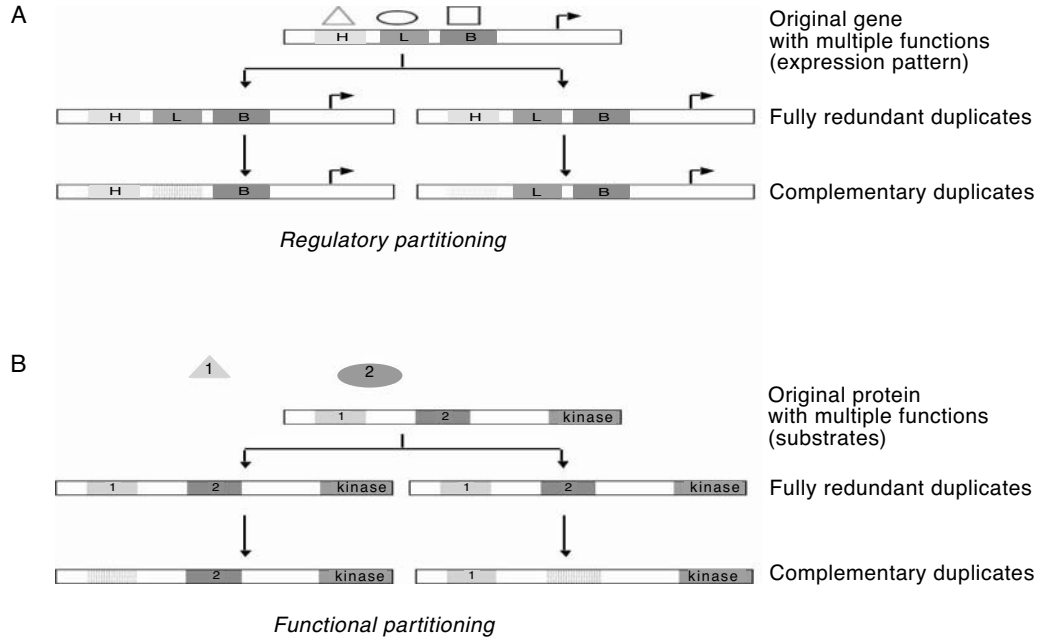

FIGURE 5.1 The duplication, degeneration, complementation (DDC) model of gene evolution. (A) *Regulatory partitioning:* The original gene is expressed in multiple cell types or tissues, such as heart (H), liver (L), and brain (B), controlled by tissue-specific transcription factors (colored shapes). After duplication, each gene copy loses regulatory sequences targeted by different transcription factors, resulting in complementary expression patterns. (B) *Functional partitioning:* The original gene encodes a protein with multiple functions, such as an enzyme that can act on different substrates (shapes 1 and 2). After duplication, residues required for substrate-specific recognition are differentially mutated, resulting in complementary loss of substrate binding. Schemes are based on the model proposed by Force, Lynch, and colleagues (Force et al., 1999; Lynch and Force, 2000). (A color version of this figure is available from the first author [mhahn@whoi.edu] upon request.)

from studies of zebrafish paralogs that have distinct patterns of expression that together sum to the expression pattern of their mammalian ortholog (reviewed by Postlethwait et al., 2004). We refer to this as *regulatory partitioning* (Figure 5.1A). For other genes, rather than (or in addition to) the partitioning of expression patterns, the fish genes may diverge with regard to specific functions—for example, differential loss of certain functional domains or specialization for subsets of ligands (*functional partitioning*; Figure 5.1B) (de Souza et al., 2005; Hawkins and Thomas, 2004; Hawkins et al., 2005). In either case, paralog-specific studies involving knock-down or other approaches applied to fish paralogs may reveal novel functional aspects of their mammalian ortholog. The possibility that fish paralogs may evolve new functions (*neofunctionalization*) must also be considered (Brunet et al., 2006; He and Zhang, 2005).

An understanding of receptor function and whether it is highly conserved, partitioned, or novel requires the application of quantitative biochemical methods that reveal the toxicologically important properties of the receptors. In the next two sections, we discuss the general principles governing ligand–receptor interactions and describe some useful experimental approaches to studying them.

Ligand–Receptor Interactions: General Principles

In physiological terms, a receptor has two properties: It recognizes a change in the environment, and it produces a response. Most cellular receptors (and nearly all of the receptors of toxicological interest) are proteins that interact with small molecule ligands. For these receptors, ligand concentration is the environmental change that must be monitored and responded to. The interaction of the receptor protein with the ligand leads to a conformational change in the protein that produces a cellular response; however, ligand binding does not automatically lead to a response, as different ligands for the same receptor may

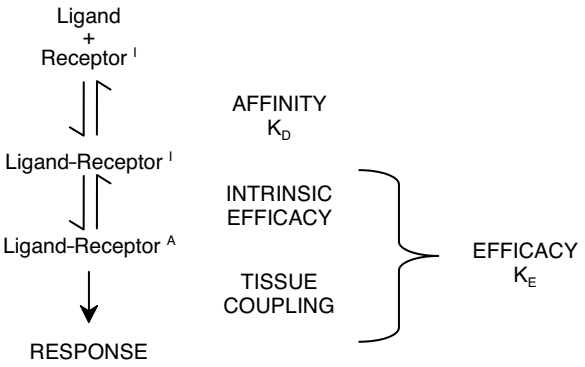

FIGURE 5.2 The pathway to receptor-mediated response. The ligand interacts with an inactive receptor ($Receptor^I$) to form a stable complex. The strength of this interaction is termed *affinity* and is typically represented by the equilibrium dissociation constant (K_D). The ability of the ligand to then convert the receptor to an active form ($Receptor^A$) is termed *intrinsic efficacy*. The magnitude of the response produced by the active receptor also depends on concentrations of other factors in the particular cell type, collectively termed *tissue coupling*. Intrinsic efficacy and tissue coupling together form the efficacy of the ligand–receptor complex, which is represented by the constant K_E in the operational model of receptor action.

differ in their ability to activate the receptor. The ligand–receptor interaction, therefore, may be thought of as a two-step process leading to activation (Figure 5.2): (1) the interaction of the ligand and the receptor (governed by a property termed *affinity*), and (2) the conversion of the receptor to an active form (a property termed *efficacy*).

Affinity

Ligand "binding" by a receptor is typically the result of several noncovalent interactions. The strength of this binding is termed *affinity*. The interaction of receptor and ligand is reversible, with rate constants for the association and dissociation of the ligand with the receptor:

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR \tag{5.1}$$

where L, R, and LR are the ligand, receptor, and ligand–receptor complex, respectively, and k_1 and k_2 are the association and dissociation rate constants. Typically association (or "on") rates are several orders of magnitude greater than dissociation (or "off") rates. At equilibrium, association and dissociation occur at an equal rate. If a single equilibrium dissociation constant (K_D) is defined to replace the rate constants:

$$K_D = \frac{k_2}{k_1} \tag{5.2}$$

then we can derive the following expression relating ligand concentration and the fraction of receptor that is occupied by ligand:

$$\frac{[LR]}{[R_T]} = \frac{[L]}{[L] + K_D} \tag{5.3}$$

where R_T is the total amount of receptor in the cell. This relationship is a variation of the Langmuir binding isotherm (Langmuir, 1916).

From Equation 5.3, it is evident that K_D is equivalent to the ligand concentration at which half the receptor is in a complex with ligand. At lower values of K_D, less ligand is needed to occupy the receptor, indicating a stronger affinity of the receptor for the ligand. Because on rates are much faster than off rates, the value for K_D is typically in the micromolar to picomolar range.

The relationship expressed by Equation 5.3 involves several assumptions about the nature of the association. The most important assumptions for the experimental measurement of binding affinity are:

- The system must have reached equilibrium; that is, association and dissociation are occurring at equal rates when binding is measured, and the fraction of ligand bound has reached a steady state. Generally, this must be determined empirically by measuring binding at a single ligand concentration over several time points.
- Binding of one ligand molecule does not effect binding of another, which includes two major components: (1) Removal of a ligand molecule from the available pool by binding does not alter the concentration of free ligand; that is, the fraction of total ligand that is bound to the receptor is relatively small. (2) Binding is not cooperative, an assumption that is likely to be violated in instances where multiple ligand molecules bind to a single receptor or where receptor proteins form complexes and will often result in nonlinear Scatchard plots (see below).

Deviation from these assumptions can be accommodated by modifications of Equation 5.3 which fall outside the scope of this chapter but are described elsewhere (Kenakin, 1999; Kenakin et al., 1992).

Measurement of Affinity

Although binding assays utilizing fluorescence techniques are seeing increased use in pharmacological research (Hovius et al., 2000), binding affinity is most commonly measured in prepared cells or tissues of interest using radiolabeled ligands. The goal of such ligand binding assays is twofold. First, free ligand must be separated from bound ligand. Second, ligand bound to the receptor must be separated from that bound to nonspecific binding sites within the cell or tissue. Several procedures to achieve the first goal are discussed in the next section. To achieve the second goal, binding assays assume that nonspecific binding is of lower affinity and cannot be saturated in the range of ligand concentrations used. In contrast, the specific receptor binds ligand with much higher affinity but is present in vastly smaller quantities, which can be saturated at relatively low ligand concentrations. Labeled ligand can be displaced from high-affinity, low-capacity receptors but not from low-affinity, high-capacity nonspecific binding sites by an excess of unlabeled ligand; therefore, the definition of high-affinity (specific) binding sites (i.e., receptors) is binding sites that can be saturated within the concentrations of ligand used. High-affinity binding is determined by subtracting the amount of ligand bound in the presence of unlabeled competitor from the amount of ligand bound in the absence of competitor.

This means that the actual data collected from an experiment consist of two binding plots, a *total binding* curve measuring binding at several ligand concentrations in the absence of competitor and a *nonspecific binding* curve determined in the presence of competitor. The *specific binding* curve is simply the difference between these two. It is important to note that specific binding in radioligand experiments is not a directly determined value, but a derived one.

Possessing both a method to determine specific binding and a theoretical relationship to describe it (Equation 5.3), it should be possible to fit data to the relationship. By measuring the amount of receptor occupied at several different free ligand concentrations, the values of R_T and K_D can be determined. First, it is necessary to determine whether sufficient data have been collected to perform a proper binding analysis. The data should include several points at concentrations of free ligand above the K_D. This can be assessed visually by plotting specific binding vs. free ligand concentration on a semilog plot but not with a linear scale of free ligand, which can be misleading (Munson and Rodbard, 1983). Figure 5.3 shows two plots of the same idealized specific binding curve. The data on the left are plotted with a linear *x*-axis and seem to indicate that the last few points are beyond the inflection point (i.e., K_D) of the curve and that a saturating ligand concentration is being approached; however, the semilog plot on the right reveals that the inflection point has not been reached, and R_T and K_D (hereafter collectively called the *binding constants*) cannot be estimated properly. The experiment must be repeated with additional treatments using higher concentrations of ligand.

When sufficient data have been collected, the binding constants can be determined. The classic method for estimating these values, and one still in use, is the Scatchard plot (Scatchard, 1949). Because Equation

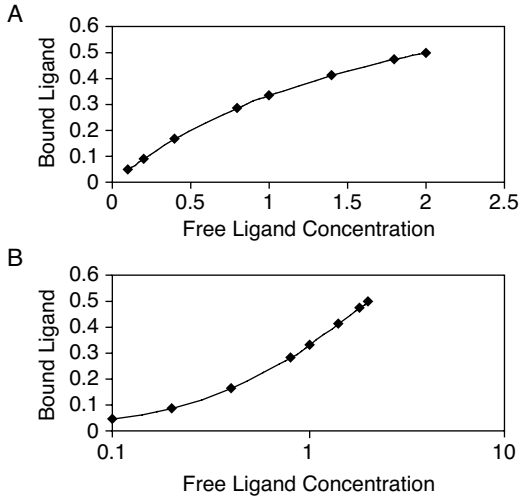

FIGURE 5.3 Achieving saturating concentrations of labeled ligand. The same values of ligand bound to receptor are represented in linear (A) and semilog (B) plots of ligand concentration. Although the receptor appears to be nearly saturated in A, the semilog plot shows that that insufficient data have been collected.

5.3 describes a nonlinear function of ligand concentration, a transformation that yields a linear relationship would be both visually satisfying and simpler to fit. By cross-multiplying the terms of Equation 5.6 and dividing throughout by [L]*K_D, such a linear relationship is established:

$$\frac{[LR]}{[L]} = -\frac{[LR]}{K_D} + \frac{[R_T]}{K_D} \tag{5.4}$$

A plot of bound ligand (or receptor) concentration divided by free ligand concentration on the *y*-axis and bound ligand concentration on the *x*-axis yields a line with a slope of $-1/K_D$ and an *x*-intercept of R_T (Figure 5.4). An obviously nonlinear relationship for data plotted in this fashion is a sign that one of the assumptions listed for Equation 5.3 is false.

Although the Scatchard plot is a useful tool for visually assessing the nature of the binding interaction and easily computing R_T and K_D, it does have its drawbacks. The transformation necessary to achieve the linear relationship includes both independent (ligand concentration) and dependent (binding) variables in the *y*-axis; therefore, data points from different ligand concentrations are weighted unequally, with points at higher ligand concentrations receiving greater relative weight in the determination of the binding constants. This typically leads to overestimation of both R_T and K_D.

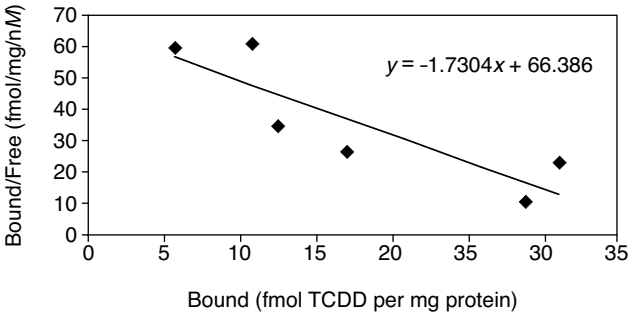

FIGURE 5.4 Scatchard plot of ligand binding. The linear fit of binding data is shown, producing estimates of 0.58 n*M* for K_D and 38 fmol TCDD per mg protein for R_T.

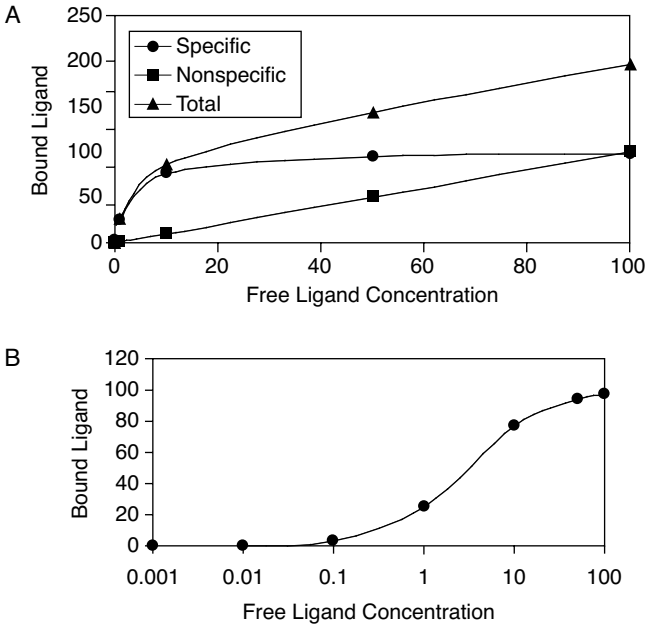

FIGURE 5.5 Idealized ligand binding data. (A) A typical plot of concentration-dependent total and nonspecific binding. Specific binding is calculated as the difference between total binding and nonspecific binding. (B) Specific binding from A is shown on a semilog plot to demonstrate that saturating concentrations of ligand are being approached.

Using computer software for performing nonlinear curve fits, it is possible to fit data directly to the Langmuir isotherm (Equation 5.3) without transformation. This produces more accurate estimates of the binding constants. The specific binding values are not measured directly, however, so this approach is still somewhat removed from the ideal of determining binding directly from measured quantities.

Nonspecific binding is by definition unsaturable within the range of ligand concentrations used, and it typically increases as a linear function of ligand concentration:

$$\mathrm{NSB} = \mathrm{m[L]} \tag{5.5}$$

where NSB is nonspecific binding and m is the slope of the line fit to the data. Total binding (TB) should simply be the sum of the specific and nonspecific binding:

$$\mathrm{TB} = \frac{[\mathrm{L}][\mathrm{R_T}]}{[\mathrm{L}] + \mathrm{K_D}} + \mathrm{m[L]} \tag{5.6}$$

Therefore, the data collected for the total binding curve can be fit to Equation 5.6 to determine the binding constants and the slope of the nonspecific binding line. Theoretically, even more accurate estimates of the binding constants can be achieved by simultaneously fitting the total binding data to Equation 5.6 and the nonspecific binding data to Equation 5.5. Idealized data showing the typical relationship among total, specific, and nonspecific binding are shown in Figure 5.5.

The method used to fit the binding data is critical when data are of relatively poor quality, which may be due to factors beyond the experimenter's control (e.g., unstable receptor or ligand, difficult tissue preparations). When the collected data closely fit Equations 5.3, 5.5, and 5.6, the binding constants estimated by the different methods are nearly identical. This is illustrated in Figure 5.6, where the data in panel A were collected from binding of [^{3}H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]-TCDD) to hepatoma cells from the fish *Poeciliopsis lucida* (PLHC-1), which have a relatively unstable aryl hydrocarbon receptor; the data in panel B were collected using the same technique from dolphin kidney cells (CDKs), which have a relatively stable receptor. Fitting the data to Equations 5.3, 5.4, or 5.5 and 5.6 (simultaneously)

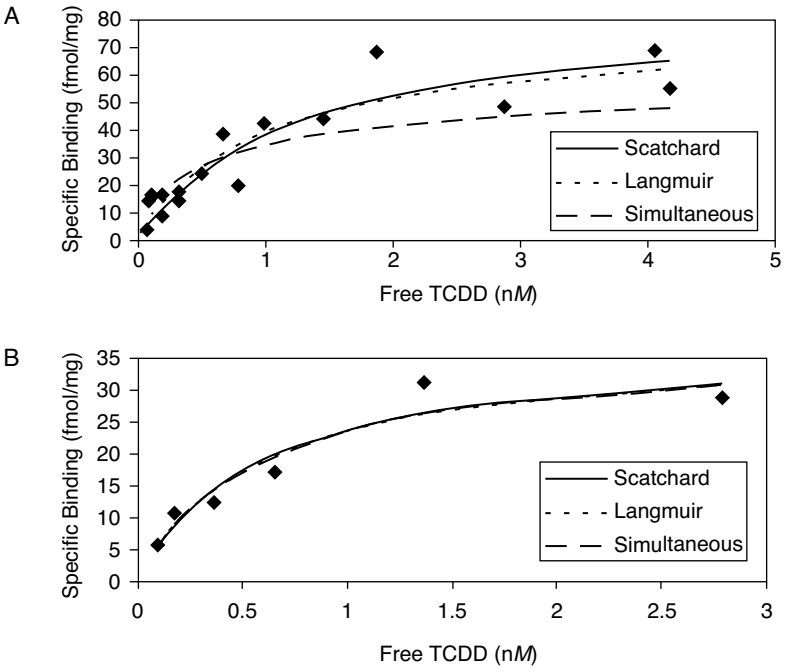

FIGURE 5.6 Effect of data quality on curve fits. Specific binding of [^{3}H]-TCDD was measured in PLHC-1 fish hepatoma cells (A) and CDK dolphin kidney cells (B), and the data were fit using the Scatchard method (Equation 5.7), directly fit to the Langmuir binding isotherm (Equation 5.6), or by simultaneous fitting of the total and nonspecific binding data (Equations 5.9 and 5.10). Binding curves predicted using the constants determined from the fits (Table 5.2) are plotted. The CDK cells were generously provided by Dr. Michael Carvan, and the data are shown with his permission.

yielded quite different estimates of K_D and R_T for PLHC-1 cells but nearly identical estimates for CDK cells (Table 5.2). Notice that as the theoretical appropriateness of the fits increases (moving down the table), the K_D and R_T decrease. Overestimation of the R_T is common when a few points with higher specific binding skew the fit, as the two points near 70 fmol/mg of specific binding do for the PLHC-1 data.

Efficacy

Interaction with a ligand is only the first step in the physiological role of the receptor. Ligand binding leads to a change in protein conformation that may activate the receptor (often referred to as receptor *activation* or receptor *transformation*). Depending on the specific receptor involved, activation may involve enzyme (e.g., protein kinase) or transport (e.g., ion channel) activity by the receptor protein itself, interaction with DNA response elements, or (most frequently) interaction with other proteins that help transduce the receptor signal. The structure of the ligand–receptor complex will vary depending on

TABLE 5.2

Binding Constants Predicted Using Different Fit Models

	Fit Model	K_D (n*M*)	R_T[a]
PLHC-1	Scatchard	1.22	84
	Langmuir	0.95	77
	Simultaneous	0.50	53
CDK	Scatchard	0.57	37.6
	Langmuir	0.58	37.5
	Simultaneous	0.57	37.1

[a] Expressed as fmol [^{3}H]-TCDD bound per mg cell protein.

the ligand bound, which produces differences in the activity of the receptor. The relative ability of the ligand–receptor complex to produce a downstream response is termed *efficacy*.

Measurement of efficacy is complicated by the fact that it depends not only on the ligand and receptor but also on the presence of other factors in the cell; for example, the same ligand for a G-protein-coupled receptor may produce varying responses in different tissues depending on the identity and concentration of G proteins present. Even within a single tissue type, efficacy varies among responses. Perhaps the best-studied example of differential efficacy among tissues and responses is the effects of the hormone estrogen and synthetic estrogen receptor ligands, termed *selective estrogen receptor modulators* (SERMs), which recapitulate different subsets of estrogenic responses (MacGregor and Jordan, 1998). The mechanism of differential efficacy has been established for the SERM tamoxifen, which induces a receptor conformation that does not facilitate interaction with transcriptional coactivator proteins (Shiau et al., 1998).

The response produced by the ligand also varies among cell types due to differences in receptor concentration. Some cells may possess receptor reserve (i.e., "spare" receptors), such that only a fraction of the receptors must be occupied to produce a maximal response, while other cells contain just sufficient receptors to produce a maximal response, and others so few that a maximal response is never produced, regardless of ligand concentration.

All of these factors contribute to the observed efficacy of a ligand, which is determined by dose–response studies. Ligands can be classified based on the responses they produce as full agonists (which produce the maximal response possible), partial agonists (which produce a less-than-maximal response, even at high ligand concentrations), and antagonists (which interact with the receptor but produce no response). Because of tissue-specific differences in the concentration of receptors and other necessary factors, the same ligand could be a full agonist in one tissue, a partial agonist in another, and an antagonist in a third. Also, partial agonists can vary widely in the amount of response they produce. For these reasons, it can be useful to compare a set of ligands in terms of their relative efficacies rather than applying conditional (i.e., tissue-dependent) labels such as "full agonist" or "partial agonist."

Several models have been advanced to describe the contribution of efficacy to response. The operational model of Black and Leff (1983) has the advantage of representing efficacy in a single term, K_E, analogous to the representation of affinity with K_D. The operational model assumes a hyperbolic relationship between the amount of ligand-occupied receptor and response:

$$\frac{E}{E_{max}} = \frac{[LR]}{[LR] + K_E} \tag{5.7}$$

where E and E_{max} are the response produced and maximal response. Substituting Equation 5.3 into Equation 5.7 produces:

$$\frac{E}{E_{max}} = \frac{[L][R_T]}{K_D K_E + ([R_T] + K_E)[L]} \tag{5.8}$$

If the binding affinity of the ligand and the concentration of receptors is known for the system, K_E can be determined by fitting dose–response data to Equation 5.8. Equation 5.8 can be solved for the conditions when response is half maximal (i.e., the EC_{50} for the system and ligand):

$$EC_{50} = \frac{K_D}{1 + ([R_T]/K_E)} \tag{5.9}$$

It is important to note that K_E depends both on the properties of the ligand–receptor complex and on properties of the tissue; however, if response is measured for a series of compounds in a single cell type, then differences among K_E values are due solely to the interaction of the ligand and receptor (Hestermann et al., 2000). Also, K_E is a useful value because it carries the same units as $[R_T]$, so $K_E/[R_T]$ is the fraction of receptors that must be occupied for a half-maximal response, giving a ready estimate of receptor reserve.

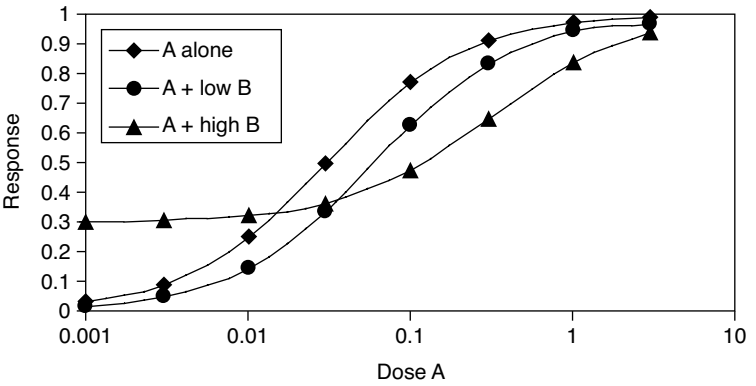

FIGURE 5.7 Effect of a low-efficacy ligand on the dose–response relationship for a high-efficacy ligand. Dose–response curves are shown for ligand A alone or with ligand B. The amount of ligand B added is the same at all doses of A within a single curve. At a low concentration of B, B alone evokes no response but antagonizes the action of A. At a higher concentration of B, B alone produces a small response and further antagonizes the action of A.

Equations 5.8 and 5.9 make clear that response depends on the concentrations of ligand and receptor as well as the affinity and efficacy constants. This takes us back to the concept expressed in Figure 5.2, that the ability of a compound to produce a response depends both on affinity and efficacy. If several compounds vary in their EC_{50} values for a response mediated by the same receptor, it is impossible to know without further information whether the compounds vary in their affinity or efficacy, or both.

Ligand-binding experiments are typically more difficult to perform than dose–response studies. Fortunately, it is possible to determine the relative efficacies of two ligands without knowing their receptor binding affinities by treating with combinations of the two. If ligand B has significantly lower efficacy than ligand A, then in combination ligand B would be expected to reduce the effect of ligand A (because it is occupying receptors without activating them). Sample data from this type of experiment are shown in Figure 5.7. A low dose of B, insufficient to elicit a response on its own, nevertheless occupies receptors, thereby requiring more A to produce a response and shifting the dose–response curve to the right. At a higher dose of B, the compound can cause a response on its own, but it also produces a greater inhibition of the response to compound A. If A and B had similar efficacies, the doses would produce additive effects, and adding B would not shift the dose–response curve to the right.

This example illustrates the importance of understanding efficacy for effective toxicological risk assessment. Because chemicals usually occur in the environment in complex mixtures, the presence of low-efficacy ligands can actually reduce the risk associated with high-efficacy ligands. Measuring the toxicity of these ligands separately and then summing to estimate the toxicity of the mixture will result in overestimation of the potential toxicity (Walker et al., 1996; Zabel et al., 1995). Thus, mixtures including low-efficacy ligands will violate the additivity assumption in the toxic equivalency factor approach (see Chapter 21). An example of the application of these principles concerning ligand affinity and efficacy and their application in the context of fish toxicology can be found in Hestermann et al. (2000).

A special case in which the concepts of affinity, efficacy, and potency are relevant to assessing the risk of mixtures involves the situation in which each of the individual compounds in the mixture is present at a concentration less than that which causes a significant biological effect on its own. Under what conditions might one expect to see a response ("something from nothing") from such a mixture (Silva et al., 2002)? The concept of concentration addition has been used to predict the effects of such mixtures (Brian et al., 2005; Thorpe et al., 2006). Clearly, however, the response will be influenced by differences in the relative intrinsic efficacies of the compounds. Understanding the interactions of compounds with different intrinsic efficacies remains incomplete; this is an important area of ongoing research.

Ligand–Receptor Interactions and Receptor Function: Approaches and Methods

The theoretical principles presented in the preceding section provide a foundation for considering practical aspects of assessing receptor function in fishes. To understand the role of receptors in mechanisms of toxicity, it is necessary to determine: (1) the biochemical characteristics of the receptors themselves; (2) the levels, location, and timing of their expression; (3) their interaction with other proteins and DNA; and (4) the genes or processes they regulate. A variety of experimental approaches and techniques that were developed originally for use in mammalian systems also have utility in fish. Often, however, modifications are required to account for physiological and other differences between fish and mammals (e.g., poikilothermy vs. endothermy). In addition, some methods are especially suited for fish, such as those that take advantage of the externally developing, transparent embryos. In this section, we summarize a few of the key approaches and techniques that are used to investigate receptor-mediated mechanisms of toxicity in fish, with examples from the well-studied AhR system.

Ligand-Binding Assays

The objective of investigating ligand–receptor interactions is to determine the physicochemical constants that govern those interactions—principally, ligand-specific kinetic constants such as on and off rates (association and dissociation rate constants), the equilibrium dissociation constant (K_D, a measure of binding affinity), and binding capacity (R_T, also called B_{max}). The theoretical aspects of these ligand–receptor interactions have been described earlier. To perform ligand-binding assays, one needs a ligand labeled with a radioactive or fluorescent tag, a method to separate ligand free in solution from that bound to proteins, and—because pure preparations of receptor are rarely available—a way to distinguish binding of ligand to the receptor (specific binding) from its binding to other proteins (nonspecific binding). Tritium ([^{3}H])-labeled or [^{125}I]-labeled ligands are commonly used, whereas [^{14}C]-labeled compounds usually do not have sufficiently high specific activity for binding assays.

Among the several ways to separate bound from free ligand is a classical method that uses *dextran-coated charcoal* (Poland et al., 1976); the charcoal adsorbs free ligand while the dextran coating minimizes the effect of charcoal on the proteins. The amount of charcoal must be determined empirically, and for some receptors species-specific sensitivity to charcoal complicates the use of this technique (Manchester et al., 1987; Nakai and Bunce, 1995). Rather than adsorb free ligand with charcoal, other methods use *hydroxylapatite* (HAP) (Gasiewicz and Neal, 1982) or *protamine sulfate* (Denison et al., 1984) to adsorb the proteins so free ligand can be washed away. Similarly, *filter-binding assays* collect solvent-aggregated proteins on glass-fiber membranes and remove free ligand by several washes (Dold and Greenlee, 1990). The latter can be performed either with cytosols prepared by differential centrifugation or with whole cells that have been incubated with radioligand. Another classical method involves *velocity sedimentation* on sucrose gradients (Okey et al., 1979; Tsui and Okey, 1981), which separates ligand–receptor complexes based on their sedimentation coefficient; the latter is a function of the size and shape of the protein complexes. Two types of centrifuge rotors can be used for this technique. Swinging-bucket rotors allow separation along the length of the centrifuge tube but require long spins (typically >18 hours). Vertical tube rotors permit much shorter spins (~2 hours) and thus facilitate analysis of labile ligand–receptor interactions (Tsui and Okey, 1981). Finally, free and bound ligand can be separated by denaturing gel electrophoresis, but only if the ligand has been covalently linked to the receptor—for example, through use of a *photoaffinity ligand* (Poland et al., 1986).

All of the approaches described above have been used to investigate fish AhRs (Hahn, 1998), as well as a variety of other receptors such as estrogen receptors (Hawkins and Thomas, 2004; Menuet et al., 2002), androgen receptors (Wells and Van Der Kraak, 2000), and retinoid receptors (Alsop et al., 2001). In some cases, multiple methods are used together to provide complementary information. Velocity sedimentation using vertical tube rotors (Figure 5.8A–D) has been useful because it is the gentlest procedure and provides ancillary information about receptor size; however, it is quite labor intensive and thus not suitable for large numbers of samples, such as are required for generating competitive

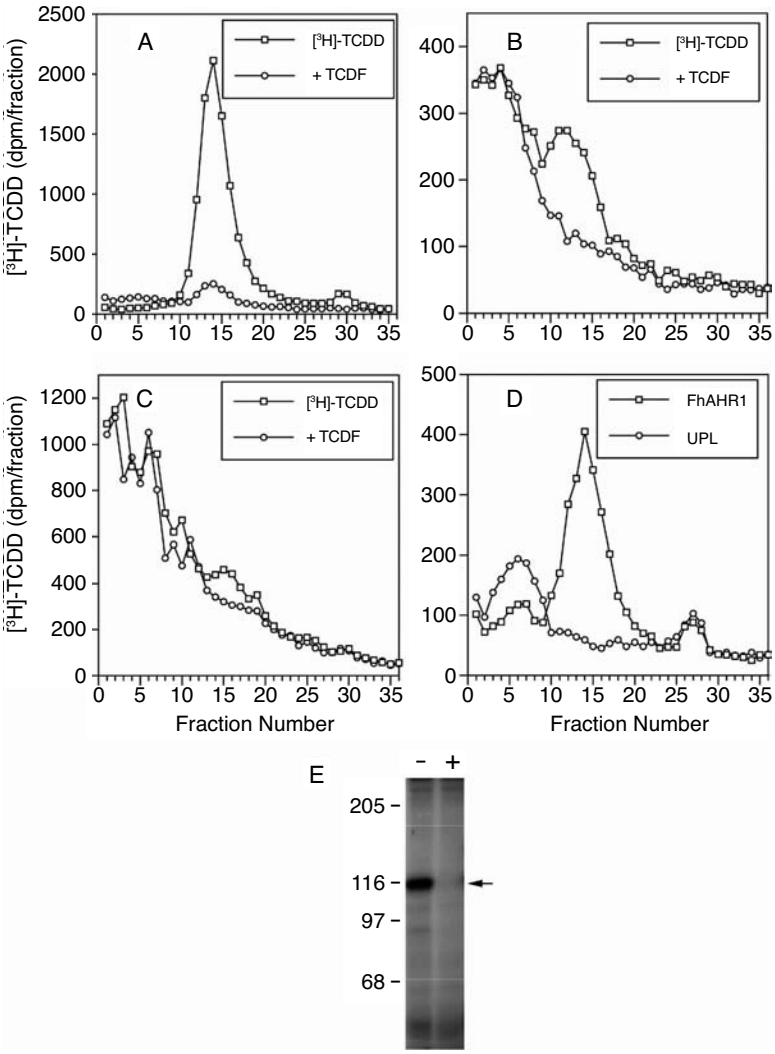

FIGURE 5.8 Data illustrating the specific binding of radioligands to AhR as measured using velocity sedimentation (A–D) and photoaffinity labeling (E). Specific binding of [^{3}H]-TCDD (~1 n*M*) to AhRs in cytosol from mouse Hepa-1 cells (A), cytosol from fish PLHC-1 cells (B), cytosol from killifish liver (C), and killifish AhR1 expressed from an AhR1 expression construct by *in vitro* transcription and translation using rabbit reticulocyte lysate (D). Samples were incubated with [^{3}H]-TCDD in the absence or presence of excess unlabeled TCDF (A–C) to determine total and nonspecific binding. In D, lysate lacking the AhR1 cDNA (unprogrammed lysate [UPL]) was used to determining nonspecific binding. (E) Photoaffinity labeling of AhRs in killifish hepatic cytosol using 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin in the absence (–) or presence (+) of excess TCDF (for details, see Hahn et al., 1994). Numbers represent molecular weight standards. Data in C were generously provided by Dr. Susan M. Bello (Bello, 1999).

binding curves. Despite the gentleness of this procedure, specific binding of [^{3}H]-TCDD to fish AhRs in cell lysates or tissue cytosol is difficult to measure as compared to mammalian AhRs (Figure 5.8A–C). In contrast, expression of fish AhRs by *in vitro* transcription and translation from cloned cDNAs produces robust peaks of specific binding in velocity sedimentation assays (Figure 5.8D); thus, this procedure has been very useful for the initial characterization of cloned fish AhR cDNAs (Abnet et al., 1999a; Andreasen et al., 2002; Karchner et al., 1999, 2005). Photoaffinity labeling (Figure 5.8E) is very sensitive and provides an estimate of receptor molecular mass but cannot be used for determining binding constants. The batch assays, such as filter-binding, hydroxylapatite, and dextran-coated charcoal, are best for

saturation binding analysis (see Figure 5.6); of these, the filter-binding assay has been most useful for studies of fish AhRs (Hestermann et al., 2000).

The assays described above are typically done using cell lysates or the soluble fraction of tissues or cells (cytosols); however, as noted above, they can also be performed on receptor proteins expressed from cloned cDNAs in bacteria, in mammalian cells, or by *in vitro* transcription and translation. An advantage of expression from cloned cDNAs is the ability to perform parallel assays in the same system but in the absence of receptor (Figure 5.8D); this provides a much more realistic assessment of nonspecific binding than that obtained by using an excess of unlabeled ligand.

Cell Culture

Cultured cells have shown great utility in characterizing receptors and receptor-dependent processes in fish, as they have in mammals. Fish cells or cell lines have been used for the direct assessment of ligand–receptor binding interactions (Hestermann et al., 2000; Lorenzen and Okey, 1990; Pollenz and Necela, 1998; Swanson and Perdew, 1991), for assessing reporter gene activation by ligand-activated transcription factors (Carvan et al., 2000), and for determining relative potencies of chemicals acting through receptor-dependent mechanisms by measuring the expression of endogenous target genes such as CYP1A (AhRs) (Clemons et al., 1996; Henry et al., 2001) or vitellogenin (ERs) (Petit et al., 1997; Smeets et al., 1999). An alternative way to characterize fish receptors is to express them in heterologous systems, such as bacteria, yeast, or mammalian cells. Cloned receptor cDNAs are inserted into an appropriate expression vector (sometimes as a fusion protein) and then are used to transform bacteria or transfect mammalian cells. Receptors expressed in this way can be characterized by the ligand-binding methods described above, as well as by reporter gene assays in which the ability of the receptor to activate transcription is assessed in the presence of different ligands. One advantage of expressing receptors in heterologous cells is that it facilitates comparative studies by providing a common cellular background on which receptors from different species can be examined (see, for example, Abnet et al., 1999b; Matthews et al., 2000).

In Vivo Assays

One of the great advantages of using fish to study mechanisms of toxicity is the ability to carry out *in vivo* experiments to assess receptor functions, especially those involved in developmental toxicity. Two of the most powerful approaches involve: (1) targeted gene knock-down to reduce or eliminate specific gene products, and (2) gene expression or overexpression by transgenesis. Currently, these are used primarily in zebrafish and other small fish models, although their use is expanding to other species as well.

Gene Knock-Down

The ability to perform targeted inactivation of mouse genes by homologous recombination (gene knock-out) has been a valuable tool in toxicological research, permitting an assessment of gene function through observation of the phenotype of animals lacking specific gene products. Gene knock-outs are not yet possible in fish. Analogous to gene knock-out, but with key differences, an anti-sense approach using morpholino-modified oligonucleotides (MOs) has been developed for producing targeted gene knock-downs in developing zebrafish (Anon., 2000; Ekker, 2004; Nasevicius and Ekker, 2000; Sumanas and Larson, 2002). Morpholino-modified oligonucleotides inhibit protein synthesis either by blocking the translational start sites of mature mRNAs (Figure 5.9A) or by altering pre-mRNA splicing (Figure 5.9B). MOs targeted to the 5′-UTR inhibit translation of maternal and zygotic transcripts by binding to mRNA between the 5′ cap and the start codon (Ekker and Larson, 2001; Summerton, 1999; Summerton and Weller, 1997); MOs targeted to exon–intron splice sites block the processing of zygotic (but not maternal) RNA (Draper et al., 2001; Ekker and Larson, 2001). By targeting the splice donor or splice acceptor site (or both), one can obtain mis-spliced mRNAs that have skipped exons or retained introns or reveal cryptic splice sites. If these modified splice products cause a frameshift or deletion of a critical part of the encoded protein, the product is inactive and the embryos are deficient for this protein. Incorrectly

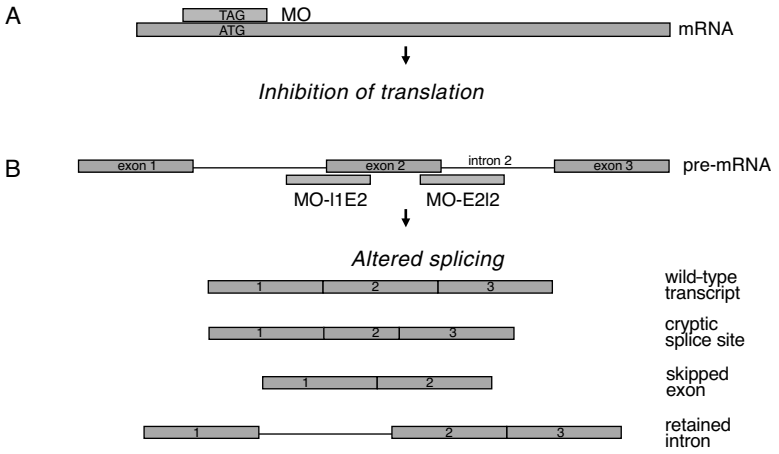

FIGURE 5.9 Two mechanisms of gene knock-down by morpholino oligonucleotides (MOs). (A) Inhibition of mRNA by MO targeted to translational start site. (B) Inhibition of pre-mRNA splicing by MOs targeted to splice donor or splice acceptor sites. For details, see text and Draper et al. (2001), Ekker (2004), and Nasevicius and Ekker (2000). (A color version of this figure is available from the first author [mhahn@whoi.edu] upon request.)

spliced transcripts can also be targeted for rapid degradation prior to translation (nonsense-mediated decay) (Baker and Parker, 2004).

Morpholino-modified oligonucleotides are injected at the one- to eight-cell stage and are distributed and retained in all cells (Ekker and Larson, 2001). They eliminate or greatly reduce the expression of the targeted protein, as indicated by expression analysis and by the fact that the phenotype of injected embryos (*morphants*) (Ekker, 2000) is in most cases indistinguishable from that of zebrafish null mutants at that locus (Lele et al., 2001; Nasevicius and Ekker, 2000) or mice bearing a null allele at the orthologous locus (Topczewska et al., 2001). MO-treated zebrafish have been shown to replicate several human genetic diseases (Nasevicius and Ekker, 2000). MOs function through at least the first 48 to 96 hours of zebrafish development, during which somitogenesis and organogenesis occur, and knock-downs lasting longer have been reported (Nasevicius and Ekker, 2000). Although used primarily in zebrafish, MOs have also been used successfully in other fish, including trout (Boonanuntanasarn et al., 2002) and lamprey (McCauley and Bronner-Fraser, 2006). The morpholino approach is proving extremely useful for identifying gene function during development, and it can be accomplished more quickly and with less cost than targeted disruption of murine loci. Similarly, gene knock-downs are finding application in developmental toxicology (Carney et al., 2004; Incardona et al., 2005, 2006; Linney et al., 2004b; Prasch et al., 2003). For example, studies using MOs have shown that AhR2 but not AhR1A and ARNT1 but not ARNT2 are required for TCDD developmental toxicity in zebrafish (Prasch et al., 2003, 2004, 2006).

Another approach for gene targeting in zebrafish is target-selected inactivation, in which zebrafish generated using mutagenized sperm are screened for point mutations that result in null alleles at specific loci. In contrast to mouse knock-outs, in which genes are targeted for homologous recombination, target-selected inactivation involves random mutagenesis followed by screening a large number of individuals for the desired mutation. The screening process is accomplished by high-throughput resequencing or by a method known as *TILLING* (Amsterdam and Hopkins, 2006; Wienholds et al., 2002, 2003) that facilitates the identification of mutated alleles. Although the use of target-selected inactivation in toxicological research has not yet been reported, this method holds great promise as a complement to MO-based knock-down approaches.

Gene knock-outs in zebrafish also have been generated by insertional mutagenesis, in which a retrovirus is used to disrupt genes at random (Amsterdam et al., 1999). The mutated gene is easily identified using viral sequences as probes. A good example of how such mutants might be used was provided by recent studies. By taking advantage of an ARNT2 mutant that was one of many mutants generated by random insertional mutagenesis (Golling et al., 2002), Prasch et al. (2004) were able to establish that ARNT2 is not required for the developmental toxicity of TCDD in zebrafish.

Transgenics

Transgenic technologies are well developed in zebrafish (Udvadia and Linney, 2003) and well suited for studying receptor-mediated mechanisms of toxicity. Transient and stable (germline) expression of transgenes can be used to screen for chemical effects on gene expression (Blechinger et al., 2002; Perz-Edwards et al., 2001), test promoter function (Jessen et al., 1998; Long et al., 1997), and map regulatory elements (Barton et al., 2001; Meng et al., 1997, 1999), all *in vivo*. Heterologous promoters and proteins have been shown to function faithfully in zebrafish, recapitulating native expression patterns (Barton et al., 2001) or rescuing mutant phenotypes (Porcher et al., 1999). The coupling of green fluorescent protein (GFP)-based reporters (Amsterdam et al., 1995; Finley et al., 2001; Gibbs and Schmale, 2000) and transparent zebrafish embryos provides a powerful system for visualizing *in vivo* gene expression. Transgenic fish also can be used to investigate gene function by assessing the phenotype of fish in which specific proteins have been overexpressed through injection of mRNA or DNA. Such gain-of-function experiments provide a valuable complement to loss-of-function approaches such as MO-based gene knock-downs (Malicki et al., 2002). One advantage of overexpression is its flexibility to test the function of heterologous proteins (i.e., from other species) and the ability to test the effect of specific mutations on protein function. Gain-of-function experiments have been used to examine the effects of overexpressing hypoxia-inducible factor-1α (HIF-1α) (Kajimura et al., 2006), estrogen receptor-related receptor α (ERRα) (Bardet et al., 2005), CYP26D1 (Gu et al., 2006), and ARNT2X (Hsu et al., 2001).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) has found widespread utility as a method for measuring the ability of receptors, other transcription factors, and associated protein complexes (including coactivators and chromatin-modifying enzymes) to occupy gene promoter and enhancer sequences *in vivo*. ChIP is replacing gel mobility shift assays as the preferred method for measuring protein–DNA interactions. ChIP has been applied most widely in mammalian cell culture, but it also has been used with fish cells (Dann et al., 2004; Hirayama et al., 2005) and embryos (Havis et al., 2006).

Genomics and Gene Expression Profiling

The emergence of genome-scale approaches and techniques has provided new opportunities for progress in a variety of fields, including comparative toxicology. Genomic approaches can be classified in a variety of ways; one useful distinction is between structural genomics and functional genomics, which provide distinct yet complementary information of relevance to mechanistic toxicology.

Structural Genomics

Structural genomics concerns gene sequences (coding and noncoding), gene structure, and gene organization—information that is usually obtained through whole-genome sequencing efforts. Genome sequences permit the description of the complete set of genes in a particular gene family, illuminating phylogenetic aspects of gene family diversity and providing information to help distinguish orthologous and paralogous genes among species. As an example, the genome sequences of the pufferfish *Takifugu rubripes* (Aparicio et al., 2002) and *Tetraodon nigroviridis* (Jaillon et al., 2004) have been useful in defining the complete sets of cytochrome P450s (Nelson, 2003) and nuclear receptors (Maglich et al., 2003) in fishes. The identification of several AhR genes in the *Fugu* genome helped resolve the orthologous and paralogous relationships between fish AhR1 and AhR2 forms (Karchner and Hahn, 2004; Karchner et al., 2005). In addition, sequenced genomes allow the identification of conserved noncoding sequences involved in regulating gene expression (Dickmeis et al., 2004). This latter approach has not yet been widely applied in fish toxicology but has great potential for understanding the regulation of toxicologically important genes.

Functional Genomics and Proteomics

The term *functional genomics* refers primarily to genome-scale assessment of gene and protein expression and interactions. There are several approaches for this; each has its own advantages and disadvantages.

A detailed description of these can be found elsewhere (Ankley et al., 2006; Cossins and Crawford, 2005; Denslow et al., 2005; Ju et al., 2007; Larkin et al., 2003b); here, we briefly describe some of these approaches and discuss their application to fish toxicology.

Several polymerase chain reaction (PCR)-based methods are available to assess differential gene expression—for example, between control and chemically treated fish. Methods such as differential display PCR (ddPCR), suppressive subtractive hybridization (SSH), and representational difference analysis (RDA) are considered unbiased in that they involve no *a priori* selection of target genes and therefore can be used for gene discovery. These methods can identify genes that are either induced (upregulated) or repressed (downregulated), but all three have high rates of false positives; thus, genes identified as differentially expressed by these methods must be confirmed by more robust assays such as real-time reverse transcription PCR (RT-PCR). In some cases, genes identified by ddPCR, SSH, or RDA are used to construct a macroarray or microarray for subsequent use in evaluating gene expression in a larger number of samples (see below). Both ddPCR and SSH have been used to reveal differential gene expression in fish exposed to toxicants (Table 5.3).

Other methods for unbiased discovery of differentially expressed genes involve high-throughput analysis of transcript abundances in two different samples. Two powerful techniques are serial analysis of gene expression (SAGE) (Velculescu et al., 1995) and massively parallel signature sequencing (MPSS) (Brenner et al., 2000), both of which provide short sequence tags of 20 to 21 bp that are usually unique and can be mapped to genome sequences to determine the genes from which they came. Both SAGE and MPSS are quantitative in that tag abundances (the number of times each tag appears) are directly related to transcript abundances in the original samples. Although there is one report of SAGE applied to fish (Knoll-Gellida et al., 2006), neither SAGE nor MPSS has yet been used in the context of fish toxicology. In addition to SAGE and MPSS, the recently developed 454 parallel sequencing technology (Emrich et al., 2007; Margulies et al., 2005; Sogin et al., 2006) is likely to be even more powerful for transcriptional profiling in a variety of applications, including fish toxicology.

The first use of microarrays (DNA chips) in fish was by Gracey et al. (2001), who created custom cDNA microarrays to measure the transcriptional response of the goby (*Gillichthys mirabilis*) to hypoxia. Microarrays (cDNA and oligonucleotide) and macroarrays are now widely used in fish biology, including toxicology. Most of the available microarray resources are targeted to zebrafish (Handley-Goldstone et al., 2005; Linney et al., 2004a; Mathavan et al., 2005; Ton et al., 2002), salmonids (Rise et al., 2004; von Schalburg et al., 2005; Vuori et al., 2006), flounder (Williams et al., 2003), carp (Cossins et al., 2006; Gracey et al., 2004), or *Fundulus* (Oleksiak et al., 2001, 2002).

Several recent reports illustrate the power of microarray-based transcriptional profiling in fish to provide insight into mechanisms of toxicity or to identify candidate biomarkers of exposure or effect (Table 5.3). In one study, the mechanism of valproic acid (VPA) teratogenesis was investigated in zebrafish embryos. Gene expression profiles after VPA exposure were similar to those observed after exposure to inhibitors of histone deacetylase (HDAC), suggesting that HDAC inhibition plays a role in VPA teratogenesis (Gurvich et al., 2005). Several groups have used microarrays to investigate the effects of TCDD. Handley-Goldstone et al. (2005) found that CYP1A was the gene most strongly induced in whole zebrafish embryos exposed to TCDD early in development, confirming the dominance of this widely studied response to TCDD and other AhR agonists. More interestingly, these authors also measured altered expression of genes encoding components of cardiac muscle sarcomeres, including myosin and troponin T2; these changes suggest an explanation for cardiomyopathy seen in fish and other vertebrates (Handley-Goldstone et al., 2005). Altered gene expression has also been measured directly in hearts of larval zebrafish exposed to TCDD, demonstrating distinct responses in heart as compared to the rest of the larvae (Carney et al., 2006). Among the changes in cardiac gene expression occurring prior to signs of cardiovascular toxicity were increases in genes encoding xenobiotic-metabolizing enzymes (CYP1A, CYP1B1, CYP1C1, sulfotransferase) and those involved in cell signaling. Organ-specific changes in gene expression also were seen in medaka exposed to TCDD (Volz et al., 2005, 2006). Dramatic differences in the direction of change were seen between liver (changes dominated by induction) and testis (many genes repressed). This study also demonstrated how gene expression profiling can be fruitfully combined with histopathological analysis, an example of "phenotypic anchoring" of microarray data (see also Luo et al., 2005; Moggs, 2005; Moggs et al., 2004; Paules, 2003). Gene

expression profiling has also provided clues to the mechanism by which TCDD inhibits fin regeneration in zebrafish. In addition to induction of genes encoding xenobiotic-metabolizing enzymes, regenerating fins of zebrafish exposed to TCDD displayed dramatically reduced expression of genes encoding extracellular matrix components and of *sox9b*, which may be an important regulator of the initial stages of regeneration (Andreasen et al., 2006). Together, these examples illustrate the emerging value of gene expression profiling in mechanistic toxicology.

In contrast to the rapidly increasing application of microarrays in fish toxicology, the use of proteomic techniques has lagged until recently (Bosworth et al., 2005; Denslow et al., 2005; Knoll-Gellida et al., 2006; Link et al., 2006a,b; Walker et al., 2007). As with DNA microarrays, proteomic studies have the power to illuminate mechanisms as well as to identify markers of exposure or effect in a way that complements RNA- and DNA-based methods. Proteomics and metabolomics also can be used to complement histopathological analyses, in a phenotypic anchoring approach that parallels that described above for microarrays (Stentiford et al., 2005; Ward et al., 2006).

The Aryl Hydrocarbon Receptor Signaling Pathway

Of all the receptors that are known as targets of environmental chemicals in fishes, two have been studied in greatest detail: aryl hydrocarbon receptors and estrogen receptors. Here, to illustrate the role of receptors in fish toxicology, we provide an overview of the AhR signaling pathway, AhR diversity, and studies demonstrating the mechanistic role of AhRs in the toxicity of planar halogenated aromatic hydrocarbons (PHAHs, or dioxin-like compounds) and polynuclear aromatic hydrocarbons (PAHs) in fishes. Additional details can be found in recent reviews of the AhR pathway in mammals (Ma, 2001; Nebert et al., 2004; Puga et al., 2005; Schmidt and Bradfield, 1996) and fishes (Hahn, 2002; Hahn et al., 2005, 2006a; Tanguay et al., 2003).

Much of what we know about the AhR and its associated signal transduction pathway has come from studies in mammalian systems—primarily murine and human cells and tissues. Although not as extensive, studies in fishes have shown that the essential features of AhR signaling in mammals are conserved (Pollenz and Necela, 1998; Pollenz et al., 2002; Wentworth et al., 2004). AhR proteins are localized primarily in the cytoplasm of cells, in association with Hsp90 and other proteins. Upon binding of ligands, AhR proteins are preferentially translocated to (or retained in) the nucleus, where they form dimers with AhR nuclear translocator (ARNT) proteins. The ligand–AhR–ARNT complex interacts with AhR response elements (AhREs; also known as XREs or DREs) to activate or repress gene expression from target genes. Diagrams of the AhR pathway can be found in recent publications (Hahn et al., 2005, 2006a).

Fish AhRs appear to have ligand structure–activity relationships similar (but not identical) to those of mammalian receptors, with high-affinity binding of TCDD, non-*ortho*-substituted PCBs, and some PAHs and lower affinity binding of other halogenated and nonhalogenated ligands such as indoles (Abnet et al., 1999b; Hestermann et al., 2000, unpublished results). Similarly, the AhR recognition sequence (AhRE) in fish is similar to that of mammals, as suggested by the ability of fish AhRs to recognize mammalian AhREs. Overlap also occurs in the identity of at least some AhR target genes in fish and mammals. CYP1A, CYP1B, and certain other genes encoding xenobiotic-metabolizing enzymes are inducible by TCDD in both groups (Stegeman and Hahn, 1994). Also inducible in mammals and fish is the AhR repressor (AhRR) gene, which encodes a repressor of AhR function (Evans et al., 2005; Karchner et al., 2002; Mimura et al., 1999; Roy et al., 2006).

Despite the mechanistic similarities, the piscine AhR pathway differs from that of mammals in some fundamental ways. One key distinction is in the number of AhR isoforms. Mammals have a single AhR, whereas most fish possess multiple (two to six) distinct AhR genes (Hahn, 2002; Hahn et al., 2006a). Phylogenetic analysis of teleost AhRs shows that there are two types, AhR1 and AhR2, which arose by a gene duplication occurring early in vertebrate evolution, prior to the divergence of fish and mammalian lineages (Hahn et al., 1997; Karchner et al., 1999). Fish AhR1 forms are orthologous (or co-orthologous) to mammalian AhRs, whereas AhR2 forms are found in fish (and birds) but not in mammals. Fish AhR1 and AhR2 have different tissue-specific patterns of expression, suggesting different functional roles. This

TABLE 5.3

Examples of Studies Investigating Differential Gene Expression in Fish Exposed Experimentally or Environmentally to Toxicants

Species and Stage	Toxicant or Comparison	Method (Number of Genes Represented)	Examples of Genes or Trends Identified	Refs.
Sheepshead minnows (*Cyprinodon variegatus*)	17β-Estradiol, 17α-ethynyl estradiol, diethylstilbestrol, methoxychlor, *p*-nonylphenol, endosulfan	ddPCR, macroarray (30–250)	Estrogens and xenoestrogens induce similar profile of altered gene expression; vitellogenin (+), choriogenin (+), zona radiata proteins (+), ERα (+), transferrin (–)	Knoebl et al. (2006), Larkin et al. (2003a)
Largemouth bass (*Micropterus salmoides*)	17β-Estradiol (E2), 4-nonylphenol (NP), 1,1-dichloro-2,2-*bis* (*p*-chlorophenyl) ethylene (*p*,*p*′-DDE)	ddPCR, macroarray (132)	E2 and NP cause similar but not identical gene expression profiles; *p*,*p*′-DDE causes sex-specific changes; vitellogenin (+), choriogenin (+), aspartic protease (+), transferrin (–)	Larkin et al. (2002, 2003c)
European flounder (*Platichthys flesus*) adults	Tyne estuary vs. Alde estuary	SSH, microarray (160)	CYP1A (+), UDPGT (+), elongation factor 1 (–)	Williams et al. (2003)
European flounder adults	Cadmium	SSH, microarray (500)	Induction of oxidative stress response; Cu/Zn SOD (+), thioredoxin (+), peroxiredoxin (+), GST (+), CYP1A (–)	Sheader et al. (2006)
Atlantic killifish (*Fundulus heteroclitus*) adults	Pyrene	SSH, ddPCR	CYP1A (+), retrotransposon (+), hepatocyte growth factor activator (+)	Roling et al. (2004)
Atlantic killifish adults	Anthracene	ddPCR, macroarray	CYP2N2 (+)	Peterson and Bain (2004)
Atlantic killifish adults	Chromium (III)	ddPCR	FABP (+), CYP2N2 (+)	Maples and Bain (2004)
Atlantic killifish juveniles	Arsenic	SSH, macroarray (96)	Myosin light chain (+), keratin II (+), tropomysin (+), parvalbumin (+)	Gonzalez et al. (2006)
Atlantic killifish adults	Elizabeth River, VA, vs. King's Creek, VA	ddPCR, macroarray	Factor XI (–), UDP-glucose pyrophosphorylase (–), complement components (–), glucose-6-phosphatase (+)	Meyer et al. (2005)

Winter flounder (*Pseudopleuronectes americanus*) adults	Chromium (VI)	SSH, macroarray	Complement component C3 (–), glutathione peroxidases (–), GSTA3 (–), peroxiredoxin (–)	Chapman et al. (2004)
Zebrafish (*Danio rerio*) embryos (25 hpf)	Valproic acid	Oligo microarray (Affymetrix®)	Similar to effects of HDAC inhibitor	Gurvich et al. (2005)
Zebrafish adults	Chlorpromazine	Brain-specific cDNA microarray (682)	Sex difference; more genes downregulated	van der Ven et al. (2005)
Zebrafish larvae (3 dpf)	TCDD	Heart-specific cDNA microarray (5184)	CYP1 (+), cardiac troponin, myosin (+), mitochondrial energy transfer genes (+)	Handley-Goldstone et al. (2005)
Zebrafish larvae (3 dpf)	TCDD	Oligo microarray (Affymetrix®)	Phase I/II metabolism (+), cell-cycle regulators (+), DNA replication (–)	Carney et al. (2006)
Zebrafish larvae (4 dpf)	*tert*-Butylhydroquinone (tBHQ)	Long oligo array (Agilent) (21,495)	Glutathione function and metabolism (+)	Hahn et al. (2007)
Zebrafish adults	Regenerating vs. normal fin ± TCDD	Oligo microarray (Affymetrix®) (14,900)	Phase I/II metabolism (+), extracellular matrix (+)(–), Frizzled 7a (+), sox9b (–), collagen maturation (–)	Andreasen et al. (2002)
Atlantic salmon (*Salmo salar*) fry	M74 vs. healthy	Salmonid cDNA array (1380)	Globins (–), oxidative stress regulated (+), apoptosis (+)	Vuori et al. (2006)
Medaka (*Oryzias latipes*) adults	TCDD	SSH, cDNA macroarrays (42,175)	Organ-specific profiles; downregulated genes and histopathology in testis; ependymin (+)	Volz et al. (2005, 2006)
Zebrafish adults	17α-Ethynylestradiol	Oligo microarray (Affymetrix®) (14,900)	Sterol biosynthesis genes (+), CYP51(+), igfbp1 (+), hormone and lipid metabolism (–), CYP1A (–)	Hoffmann et al. (2006)

Note: (+) = upregulated; (–) = downregulated

appears to be the case in zebrafish, in which AhR2 has been implicated as the form responsible for nearly all of the developmental toxicity of TCDD (see below). Thus, although in mammals the single AhR (AhR1 ortholog) is required for TCDD toxicity during development (Mimura et al., 1997), in zebrafish it is the AhR paralog (AhR2) that plays this role. Whether this is specific to zebrafish or is true generally in fish remains to be determined. In addition to AhR1 and AhR2, cartilaginous fishes possess a novel form of AhR designated AhR3 (Merson and Hahn, 2002). The functional characteristics of elasmobranch AhRs have not yet been established.

Some fish possess multiple forms of ARNT. Zebrafish share with mammals the presence of two ARNT genes—ARNT1 and ARNT2—and in both cases ARNT1 appears to be the toxicologically most relevant partner for AhR (Prasch et al., 2004, 2006; Sekine et al., 2006; Walisser et al., 2004). In other species of fish, only ARNT2 has been identified (Pollenz et al., 1996; Powell et al., 1999).

Recent studies using targeted knock-down of gene expression to study the toxicity of AhR ligands illustrate the power of the zebrafish model in mechanistic research. Using morpholino antisense oligonucleotides (MOs), AhR2 was shown to play a key role in the developmental toxicity of TCDD (Antkiewicz et al., 2006; Bello et al., 2004; Dong et al., 2004; Prasch et al., 2003; Teraoka et al., 2003) and in the ability of TCDD to inhibit fin regeneration (Mathew et al., 2006). The role of AhRs in the toxicity of PAHs and other nonhalogenated ligands is more complex, with both AhR2-dependent and AhR2-independent effects (Billiard et al., 2006; Incardona et al., 2004, 2005, 2006). MO-based knock-down has also helped illuminate the role of CYP1A in the toxicity of PHAHs and PAHs. Because CYP1A induction is the most well-known and most striking (in terms of magnitude) result of AhR activation, an important role for CYP1A in TCDD toxicity has been hypothesized, possibly involving the generation of reactive oxygen species (ROS) (Schlezinger et al., 1999; Teraoka et al., 2003); however, zebrafish embryos in which CYP1A expression and induction are prevented or reduced by injection of a CYP1A-MO are just as sensitive as uninjected fish to the developmental effects of TCDD (Carney et al., 2004). In contrast, CYP1A knock-down enhances the developmental toxicity of the PAH-like AhR agonist β-naphthoflavone (BNF) and PAH-containing mixtures (weathered crude oil) but provides partial protection against the toxicity of the PAH pyrene (Billiard et al., 2006; Incardona et al., 2005). CYP1A, then, appears to have a protective role with respect to some nonhalogenated compounds but is involved in the bioactivation of others.

The possible involvement of CYP induction in the toxicity of AhR ligands is complicated by the existence of other AhR-regulated CYP1 enzymes in fishes: CYP1B1, CYP1B2, CYP1C1, and CYP1C2 (Godard et al., 2005; Leaver and George, 2000; Wang et al., 2006). The role, if any, of these enzymes in PHAH and PAH toxicity has not yet been investigated.

Other Receptors and Ligand-Activated Transcription Factors

Aryl hydrocarbon receptors have been studied extensively in fishes and serve as an example of how receptors—and the genes they regulate—participate in mechanisms of toxicity and how this may be investigated using the approaches and tools available in fish biology. A variety of other receptors and other types of transcription factors activated by xenobiotics are also involved in fish toxicology (Table 5.1), although in many cases a molecular understanding is just beginning to emerge. We briefly mention a few of these other transcription factors and some key features with regard to their presence and function in fishes.

Nuclear Receptors

Aryl hydrocarbon receptors are not unique among receptors in displaying diversity among taxa. Other receptors that are targets of endocrine-disrupting compounds, such as estrogen receptors and other members of the nuclear receptor (NR) superfamily, exhibit such diversity (Baker, 2005; Bardet et al., 2002; Hawkins et al., 2000, 2005; Thornton, 2001). Although mammals possess two estrogen receptors (ERα and ERβ), fish possess an ERα and two ERβ forms (ERβa and ERβb), the result of the fish-specific genome duplication mentioned above (Hawkins et al., 2000; Menuet et al., 2002). Similarly, although mammals

possess three estrogen receptor-related receptors (ERRs), fish possess up to six ERR genes (Bardet et al., 2004; Maglich et al., 2003; Tarrant et al., 2006). The role of nuclear steroid receptors in fish endocrine toxicology is detailed in Chapters 10 and 25. In addition, recent studies have identified membrane steroid receptors that are not related to the nuclear receptors but rather are G-protein-coupled receptors that mediate rapid nongenomic actions for estrogens, androgens, and progestins (Zhu et al., 2003a,b). These receptors, which may also be targets for environmental chemicals, are also described in Chapter 10.

Other nuclear receptors of importance in toxicology include the constitutive androstane receptor (CAR) and pregnane X receptor (PXR). The discovery of these receptors in mammals and their initial characterization in fishes have illuminated a long-standing mystery in toxicology. Early studies of CYP induction in mammals had suggested the existence of two types of responses: *3-MC type* and *PB type*, named after the model inducers 3-methylcholanthrene (3-MC) and phenobarbital (PB), which induce primarily CYP1A and CYP2B, respectively. Induction of CYP1A by 3-MC was well known to occur through the AhR, but the mechanism of PB-type induction remained elusive for many years. Interestingly, fish display 3-MC-type but not PB-type induction (Addison et al., 1987; Ankley et al., 1987; Elskus and Stegeman, 1989; Kleinow et al., 1990), but it was not known whether this was caused by lack of orthologous CYP2 genes or lack of the induction mechanism (reviewed in Stegeman and Hahn, 1994). Studies in mammals identified CAR as the transcription factor regulating CYP2 induction and PXR as the regulator of CYP3A induction (although some functional overlap occurs between the gene targets of these two receptors) (Handschin and Meyer, 2003). Searches of fish genome sequences and homology cloning efforts reveal that fish possess a homolog of mammalian PXR, but CAR appears to be absent (Maglich et al., 2003; Moore et al., 2002; Bainy and Stegeman, 2004). Evolutionary studies in a variety of vertebrates suggest that mammalian CAR and PXR arose by a gene duplication in the mammalian lineage (Handschin et al., 2004; Reschly and Krasowski, 2006); thus, nonmammalian PXR homologs are related to both PXR and CAR. The zebrafish PXR has been cloned; like other vertebrate PXRs, it has a broad ligand specificity, and it is activated by many of the known activators of mammalian PXRs (Bainy and Stegeman, 2004; Moore et al., 2002). Currently, the role of PXR in fish toxicology is not well understood beyond its probable function in regulating CYP3 expression. Recent studies have shown that several compounds, including some xenoestrogens, can induce PXR expression in fish (Bresolin et al., 2005; Meucci and Arukwe, 2006; Mortensen and Arukwe, 2006).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors with a variety of roles in regulating lipid metabolism. PPARs have been studied extensively in mammalian systems; mammals have three PPAR isoforms that act as heterodimers with the retinoid X receptor (RXR). PPAR ligands include fatty acids (natural ligands), fibrate drugs, phthalate ester plasticizers, and herbicides (Grun and Blumberg, 2006; Peraza et al., 2006). Some fish species may possess additional PPAR forms as compared to mammals (Escriva et al., 2002; Hahn et al., 2005; Leaver et al., 2005; Maglich et al., 2003; Robinson-Rechavi et al., 2001). As compared to mammalian PPARs, fish PPARs have added complexity in terms of their diversity, expression patterns, and ligand specificity (M. J. Leaver, pers. commun.).

Corticosteroid receptors regulate responses to stress and salt balance, including seawater adaptation. Fish have two glucocorticoid receptors (GRs) and a mineralocorticoid receptor, whereas mammals have one of each (Bury et al., 2003; Greenwood et al., 2003; Maglich et al., 2003; Prunet et al., 2006; Stolte et al., 2006). Studies in mammals suggest that GR could be directly affected by xenobiotics (Johansson et al., 1998), but the role of fish GRs in mechanisms of toxicity is not well understood (Knudsen and Pottinger, 1999; Vijayan et al., 2005). Evidence in fish suggests interactions between GR signaling and other receptor-dependent signaling pathways, such as the AhR pathway (Celander et al., 1996, 1997; DeVault et al., 1989). For a detailed description of fish corticosteroid receptors as targets for xenobiotics, see Vijayan et al. (2005).

Other fish nuclear receptors also are potential targets for xenobiotics; these include thyroid hormone receptors (TRs), androgen receptors (ARs), and retinoid receptors (RXRs, RORs, RARs). Thyroid hormone receptors have important roles in metamorphosis and other developmental processes in fish (Power et al., 2001) and thus are likely to be important targets of contaminants, through direct or indirect mechanisms (Brown et al., 2004; Crane et al., 2005; Elsalini and Rohr, 2003; van der Ven et al., 2006). Retinoid and androgen receptors have also been examined as targets for environmental chemicals in fishes (Alsop et al., 2003; Hewitt et al., 2003; Makynen et al., 2000; Wells and Van Der Kraak, 2000).

Neurotransmitter Receptors

Fish, like other vertebrates, possess a large variety of receptors for neurotransmitters such as GABA, glutamate, acetylcholine, and serotonin, as well as other receptors such as ryanodine receptors. All of these may be targets for environmental chemicals, as described in Chapters 9 and 20 and elsewhere (Baraban et al., 2005; Carr et al., 1999; Linney et al., 2004b; Stehr et al., 2006).

Olfactory Receptors

Another group of receptors that are potential targets for xenobiotic chemicals are the chemosensory receptors used by fish to detect small molecules (amino acids, steroids, fatty acids) in their aquatic environment. These receptors include the classical odorant receptors (Alioto and Ngai, 2005), vomeronasal receptors (Hashiguchi and Nishida, 2005), and the newly identified trace amine-associated receptors (TAARs) (Gloriam et al., 2005; Liberles and Buck, 2006). Odorant receptor gene expression changes during the parr–smolt transformation in Atlantic salmon (Dukes et al., 2004). These receptors have been suggested to be important in homing behavior, through imprinting on natal odor cues (Barinaga, 1999; Dittman et al., 1997; Nevitt et al., 1994); thus, interference with olfactory function in fishes could have long-term reproductive consequences. Few studies, however, have addressed the effect of toxicants on olfactory receptor function. One study showed that exposure of salmon to copper interfered with the neurophysiological response to natural odorants (Baldwin et al., 2003), illustrating the potential toxicological significance of the olfactory system.

Other Xenobiotic-Activated Transcription Factors

Other ligand-activated transcription factors play important roles in mechanisms of toxicity in fish. Some of these do not function as classically defined receptors, in that they do not exhibit high-affinity specific binding of ligands; rather, they are activated by other types of interactions with xenobiotics. One example of such transcription factors are those in the Cap'n'Collar basic leucine zipper (CnC-bZIP) family, including NRF2 (nuclear factor erythroid-derived-2 [NFE2]-related factor 2).* NRF2 and related proteins are activated by oxidative stress and upregulate the expression of genes that are part of the antioxidant response, including several phase II biotransformation enzymes (Nguyen et al., 2003).

Fish possess an antioxidant response that is similar, although not identical, to that of mammals, as described in Chapter 6 and elsewhere (Carvan et al., 2001; Hahn et al., 2005). A fish NRF2 ortholog has been identified and shown to regulate the induction of glutathione *S*-transferase π (GSTP), NQO1, and gamma-glutamylcysteine synthetase (γ-GCS) after exposure of zebrafish embryos and larvae to *tert*-butylhydroquinone (tBHQ) (Kobayashi et al., 2002; Suzuki et al., 2005); thus, the function and target genes of NRF2 appear to be conserved in mammals and fishes. As with many other transcription factors, however, the diversity of NRF2-like genes appears to be greater in fish than in mammals (Hahn et al., 2005). Understanding the different roles of these NRF genes in the response to xenobiotic exposure is an important goal of future research. Details concerning the mechanisms of response to oxidative stress can be found in Chapter 6.

Another example of a nonreceptor, ligand-activated transcription factor is the metal-responsive transcription factor (MTF-1). MTFs are zinc finger proteins that are activated (either directly or indirectly) by heavy metals such as Zn^{2+} and Cd^{2+}, and they regulate transcription through binding to metal-responsive elements (MREs) in the 5′-regulatory region of genes such as those encoding metallothioneins (MTs) (Giedroc et al., 2001; Samson and Gedamu, 1998). MTF-1 can also be activated by oxidative stress (Dalton et al., 1996). MTF-1 has been characterized in several fish species, and it appears to function similarly in fish and mammals (Auf der Maur et al., 1999; Chen et al., 2002; Dalton et al., 2000).

* NRF2 is also known as NFE2L2 (NFE2-like-2).

Conclusions and Future Directions

Our current understanding of receptor-mediated mechanisms of toxicity in fishes is only modest, but recent technical developments and the availability of genome-scale information have greatly accelerated the rate at which progress is being made. During the next 10 years, we anticipate substantial advances in our understanding of the impact of chemicals on fishes (fish as targets) as well as new mechanistic insights of more fundamental significance (fish as models). These advances are most likely to occur in the area of developmental toxicology, especially neurotoxicology, and will be facilitated by a more complete understanding of differences between mammals and fishes in the number and diversity of receptors that are targets for chemicals.

In the next few years, genome-scale profiling (functional genomics, proteomics, metabolomics) will contribute to the description of complex regulatory networks and an understanding of how they are perturbed by chemicals. These networks will include not only receptor-dependent signaling pathways, which we currently depict essentially as linear sequences of events, but also the various cross-talk and positive/negative feedback loops that are associated with them. Understanding such interactions will be essential to assessing risks to populations exposed to multiple toxicants and other environmental stressors.

With regard to receptor-dependent mechanisms of toxicity in fishes, many important questions remain; for example, what is the role of receptors in determining sensitivity to chemicals and in explaining species- and population-specific differences in susceptibility? What is the relative importance of differences in receptor diversity (number of paralogs), protein sequence, or receptor expression? What is the role of receptor polymorphisms in differential susceptibility observed among individuals and populations (Greytak and Callard, 2007; Hahn et al., 2004; Roy and Wirgin, 1997)? What are the target genes that are regulated by receptors, and which ones are directly involved in mechanisms of toxicity? These and many other questions will continue to provide interesting challenges and opportunities to researchers interested in understanding the impact of chemicals on fishes, whether for their own sake or as models for humans.

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6

Reactive Oxygen Species and Oxidative Stress

Richard T. Di Giulio and Joel N. Meyer

CONTENTS

Introduction

Mechanisms underlying the toxicity of numerous environmental pollutants are discussed throughout this book. The purpose of this chapter is to describe a particular set of phenomena that collectively comprise an important mechanism of chemical toxicity and cellular defense. These phenomena, referred to as *oxidative stress*, apply to a diverse array of chemicals and result in a diverse array of ultimate health outcomes. The study of oxidative stress broadly includes biological phenomena associated with the generation of reactive oxygen species (ROS), molecular systems designed to protect cells from ROS (often referred to as *antioxidant defense systems*), and the deleterious impacts of ROS. An excellent detailed monograph on this subject is Halliwell and Gutteridge (1999).

As in many areas of mechanistic toxicology, understanding of oxidative stress in fishes has lagged behind understanding in mammals and, indeed, has depended to a large degree on studies in other biological systems for the development of methodologies and concepts; however, the study of oxidative stress in fishes is currently an active area of investigation that is revealing a number of important similarities and differences with other organisms. Moreover, the multitude of chemicals entering freshwater and marine systems that can contribute to oxidative stress underlies the need for information on this phenomenon in fishes. In this chapter, we describe fundamental aspects of ROS chemistry and generation, antioxidant defense systems, cellular and organismal impacts, and specific mechanisms by which pollutants play roles in these processes. We also describe the current state of understanding of oxidative stress in fishes, including comparisons with other organisms.

Reactive Oxygen Species and Free Radicals

Oxygen (as dioxygen, O_2) currently accounts for about 21% of the Earth's atmosphere, and oxygen is the most abundant element in the planet's crust (at 54%); however, life first evolved under essentially anaerobic conditions, and O_2 is believed to have first appeared as a byproduct of photosynthesis by blue-green algae (cyanobacteria) beginning about 2.5 billion years ago (Harman, 1986). As atmospheric O_2 gradually rose over the ensuing millennia, several notable developments occurred:

1. Organisms remaining anaerobic either retreated to anaerobic microenvironments or perished due to the effects of O_2 such as oxidation of metabolic intermediates (such as thiols, iron, and pteridines), enzyme inhibition (such as occurs with nitrogenases), and effects of ROS generated during oxidations.
2. Aerobic organisms evolved that took advantage of the electron-accepting capacity of O_2. The energetic efficiency of O_2 as an electron acceptor over those employed by anaerobes (iron and sulfur, for example) enhanced the evolution of more advanced life forms (i.e., eukaryotic organisms). This switch required the concomitant development of mechanisms to protect these organisms from the toxic effects of O_2.
3. Ozone (O_3) levels in the atmosphere rose, giving organisms protection from intense solar ultraviolet radiation; this also enhanced eukaryotic evolution.

Although the focus of this chapter is on toxicities associated with oxygen species, the critical role played by O_2 in shaping and promoting advanced life forms as we know them is of fundamental importance.

The primary basis for the toxicity of oxygen lies in its propensity to undergo electron transfers that yield reactive intermediates, here termed *ROS*. On the other hand, the ability of O_2 to accept electrons underlies its utility to aerobic organisms; for example, a key function of O_2 is to serve as the terminal electron acceptor in mitochondrial electron transport, which drives the production of high-energy adenosine triphosphate (ATP). In this process, O_2 is reduced to H_2O; this is a four-electron reductive process that proceeds sequentially through the one-, two-, and three-electron products. These univalent reductions of O_2 to water are shown in Equation 6.1 to Equation 6.4:

$$O_2 + e^- \rightarrow O_2^{\bullet -} \tag{6.1}$$

$$O_2^{\bullet -} + e^- \xrightarrow{2H^+} H_2O_2 \tag{6.2}$$

$$H_2O_2 + e^- \xrightarrow{H^+} \cdot OH + H_2O \tag{6.3}$$

$$\cdot OH + e^- \xrightarrow{H^+} H_2O \tag{6.4}$$

$$\text{Sum: } O_2 + 4e^- \rightarrow H_2O \tag{6.5}$$

The one-, two-, and three-electron products shown in Equations 6.1, 6.2, and 6.3, respectively, are the *superoxide anion radical*, *hydrogen peroxide*, and the *hydroxyl radical*, respectively. The two radical species are free radicals; as defined by Halliwell and Gutteridge (1999), a free radical "is any species capable of independent existence that contains one or more unpaired electrons." The existence of unpaired electrons tends to make free radicals reactive, although the range of reactivity among free radicals is extensive. $O_2^{\bullet -}$, for example, is relatively weakly reactive, while ·OH is extremely reactive and is among the most potentially deleterious compounds among radicals and ROS encountered in cells. Thus, ·OH reacts indiscriminately with cellular constituents such as membrane lipids, proteins, and DNA. Due to its reactivity, it has an *in vivo* lifetime measured in nanoseconds (Pryor, 1986). Interestingly, O_2 itself is by definition a free radical; it contains two unpaired electrons, each in a different π orbital and having parallel spins. This last feature impedes the ability of O_2 to act as an oxidant via accepting electron pairs from typical molecules containing electron pairs with opposite spins. If it were not for this spin restriction, O_2 would qualify as an extremely potent ROS, and aerobic life undoubtedly would have a very different nature.

Although H_2O_2 is not a radical, it is an important ROS. It is a weak reducing and oxidizing agent, which lends to its use as a safe disinfectant at high concentrations. Its uncharged nature permits it to cross cell membranes; however, its ability to serve as a precursor to ·OH at physiologically meaningful levels underlies its significance to cellular oxidative stress. This ability of H_2O_2 to generate ·OH via reaction with $O_2^{\bullet -}$ was first postulated by Haber and Weiss (1934), who proposed the following reaction:

$$O_2^{\bullet -} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2 \tag{6.6}$$

Reaction 6.6 is termed the *Haber-Weiss reaction*, and reaction rates in aqueous solution, although thermodynamically favorable, are very low; however, as noted by Weiss in 1935 (Halliwell and Gutteridge, 1999), this reaction can be effectively catalyzed by transition metals such as iron, as shown in Equations 6.7 and 6.8:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \tag{6.7}$$

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+} \tag{6.8}$$

$$\text{Net: } O_2^{\bullet -} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2 \tag{6.9}$$

The net reaction, Equation 6.9, is the Haber-Weiss reaction. Equation 6.8 is referred to as the *Fenton reaction* (Walling, 1975). Other transition metals present in cells, particularly copper, also can participate in Fenton-like reactions. The abilities of iron and copper to participate in cellular ·OH generation, in addition to their roles as essential nutrients, may underlie the existence of proteins (such as ferritin for iron and ceruloplasmin and metallothionein for copper) that tightly regulate their cellular transport and fate.

In addition to these three O_2 reduction products, several other ROS and related chemicals can play important roles in oxidative stress. These include singlet oxygen, ozone, alkoxyl and peroxyl radicals, and reactive nitrogen species, such as nitric oxide and peroxynitrite (Halliwell and Gutteridge, 1999). Additionally, carbon- and sulfur-centered free radicals occur and affect cellular function. Singlet oxygen (commonly denoted as $^1O_2$) is an excited state of O_2 at 22.4 kcal above ground state. In this form, the outer π electrons are paired and in antiparallel spins; thus, $^1O_2$ is not a free radical, but the spin restriction of ground-state O_2 is removed, making $^1O_2$ more reactive than O_2. A major source of $^1O_2$ in the environment is through excitation of ultraviolet-absorbing chemicals, including many polycyclic aromatic hydrocarbons (PAHs). This phenomenon has important ramifications in aquatic toxicology and is discussed later in this chapter.

Ozone (O_3) also is not a free radical but is a much more powerful oxidizing agent than O_2. Ozone is produced in the stratosphere by the photodissociation of O_2 into oxygen atoms, which then react with O_2 to produce O_3. Tropospheric (ground-level) O_3 is produced by sunlight-enhanced reactions between nitric oxides and volatile organic compounds (VOCs); warmer temperatures and fossil-fuel combustion produce nitric oxides, which enhance this process. While stratospheric O_3 provides protection to organisms by reducing the solar ultraviolet radiation that reaches the Earth's surface, ground-level O_3 can be toxic, particularly to animal respiratory systems and plant photosynthesis.

Alkoxyl (RO·) and peroxyl (ROO·) radicals are potent oxidants that often arise in cells subsequent to ·OH attack of organic chemicals. Oftentimes, such attack initially produces carbon-based radicals, which under aerobic conditions react with O_2 to produce RO· and ROO·. This process underlies membrane lipid peroxidation, a hallmark cellular toxicity associated with ROS that is described below.

Although not the focus of this chapter, reactive nitrogen species (RNS) also play important roles in cellular physiology and toxicity and interact with ROS. Nitric oxide (NO·) is synthesized in many organisms by an enzyme group known as the *nitric oxide synthases* (NOSs); these enzymes catalyze the oxidation of arginine to produce NO· and citrulline (Muriel, 2000). Three forms of NOS have been identified in mammals, referred to as type 1, constitutive, or neuronal NOS (cNOS, nNOS); type 2 or inducible NOS (iNOS); and type 3 or endothelial NOS (eNOS). There is good evidence for conservation of genes coding NOSs in other organisms, including fishes (Cox et al., 2001).

NO· has been studied predominantly from the standpoint of the normal physiological roles it plays. These include acting as mediators of phagocytosis by macrophages and neutrophils and functioning as a neurotransmitter to relax smooth muscle and thereby produce vasodilation (Ignarro, 2002; Neumann et al., 2001; Toda and Okamura, 2003). NO· is not particularly reactive with nonradicals but is highly reactive with other radicals (Halliwell and Gutteridge, 1999). Toxicities associated with NO·, observed in some instances, are thought in large part to be due to its facile reaction with $O_2^{\bullet-}$. This reaction has at least two potentially deleterious consequences: (1) the loss of functional NO·, thereby interfering with vasodilation (and potentially leading to hypertension, for example), and (2) the production of peroxynitrite via the reaction:

$$O_2^{\bullet-} + NO\cdot \rightarrow ONOO^- \tag{6.10}$$

Peroxynitrite is a very powerful oxidizing species that can elicit cytotoxicity through several mechanisms, including those described below for ROS as well as by nitration of DNA bases and aromatic amino acids (Beckman and Koppenol, 1996).

Endogenous Cellular Sources of Reactive Oxygen Species

Before describing mechanisms by which chemical pollutants can generate ROS and enhance oxidative stress, it is important to discuss mechanisms by which these species arise normally. As alluded to earlier,

the cellular generation of ROS is an unavoidable cost of aerobic life—the price exacted for making use of the energetic efficiency of O_2 as an electron acceptor.

Perhaps quantitatively the most important sources of ROS during normal cellular metabolism are the electron transport chains of mitochondria, the endoplasmic reticulum, and chloroplasts. Photosynthesis within chloroplasts poses unique problems for plants in terms of oxidative damage, which can be exacerbated by many common air pollutants (Hippeli and Ernstner, 1996). In animals, mitochondrial respiration is likely the most important source of ROS *in vivo*. It is estimated that under normal cellular levels of O_2 consumption, about 0.1% of the O_2 utilized by mitochondria forms $O_2^{\bullet-}$ (Beckman and Ames, 1998; Fridovich, 2004). Although the precise location of this leakage is not completely resolved and may depend on conditions, complex I and III constituents are often involved (Finkel and Holbrook, 2000; Turrens, 1997). Endoplasmic reticula (*microsomes* in subcellular fractions) are another important source of ROS production. Oxidative metabolism of endogenous compounds and xenobiotics by the cytochrome P450 enzyme systems that function in this fraction were discussed in detail in Chapter 4. As described, P450s catalyze oxidations by cleaving O_2, with one oxygen atom ultimately added to the substrate and the other ultimately giving rise to H_2O. In this process, two electrons provided by NADPH–P450 reductase or cytochrome b_5 are sequentially added to drive catalysis. This cycle can be uncoupled, resulting in the diversion of electrons to give rise to $O_2^{\bullet-}$ and H_2O_2 (Goeptar et al., 1995). Some xenobiotics can greatly enhance this uncoupling, as described later in this chapter.

Several oxidative enzymes can also generate ROS during catalysis, including xanthine oxidase, tryptophan dioxygenase, diamine oxidase, guanyl cyclase, and glucose oxidase (Fridovich, 1978; Halliwell and Gutteridge, 1999). Additionally, a number of molecules with key roles in cellular function can auto-oxidize in the presence of O_2, yielding $O_2^{\bullet-}$; these include glyceraldehyde, $FMNH_2$, $FADH_2$, epinephrine and norepinephrine, dopamine, tetrahydropteridines, and thiols such as cysteine (Halliwell and Gutteridge, 1999). These autoxidations are oftentimes substantially accelerated in the presence of transition metal ions such as iron and copper. The O_2-carrying proteins hemoglobin and myoglobin can also be sources of $O_2^{\bullet-}$ (Balagopalakrishna et al., 1996; Brantley et al., 1993). In these proteins, O_2 binding requires the reduced state of iron, Fe(II), in the heme group. Occasionally, oxygen will release as $O_2^{\bullet-}$ and concomitantly yield Fe(III) in the heme. These forms of hemoglobin and myoglobin are referred to as *methemoglobin* and *metmyoglobin*, respectively, and are unable to bind O_2. Approximately 3% of human hemoglobin is thought to undergo conversion to methemoglobin every day, suggesting a substantial basal flux of ROS in erythrocytes (Winterbourn, 1985). As discussed later, many chemicals can significantly increase methemoglobin formation.

In the context of oxidative stress, ROS are cast as bad actors, but it is important to note that ROS play positive roles as well. An important example of this is the production of ROS by neutrophils and macrophages by many vertebrates wherein ROS are employed in the phagocytic activity of these cells (Babior, 2000). Upon stimulation, these cells increase O_2 consumption up to 20 times resting levels; this phenomenon is referred to as the *respiratory burst*, a misnomer because it is unrelated to mitochondrial respiration. Stimulants for this burst include opsonized bacteria and zymosan (from yeast cell walls), bacterial peptides such as *N*-formylmethionylleucylphenylalanine (FMet-Leu-Phe), the lectin concanavalin A, and phorbol esters, such as phorbol myristate acetate (derived from oil of the seeds of *Croton tiglium*, a plant native to southeastern Asia). During the respiratory burst, NADPH provided by the pentose phosphate pathway is oxidized to NADP+ by NADPH oxidase, an enzyme complex associated with the plasma membrane (Henderson and Chappell, 1996); the two electrons abstracted from each NADPH are transferred to O_2, yielding $O_2^{\bullet-}$ as indicated in Equation 6.11:

$$\mathrm{NADPH} + 2\mathrm{O_2} \rightarrow \mathrm{NADP^+} + \mathrm{H^+} + 2\mathrm{O_2^{\bullet-}} \tag{6.11}$$

Because $O_2^{\bullet-}$ is not highly reactive in aqueous solution, it is considered unlikely that $O_2^{\bullet-}$ itself is the ultimate phagocytic agent employed by neutrophils and macrophages (Halliwell and Gutteridge, 1999); however, $O_2^{\bullet-}$ can readily react with itself and dismutate into hydrogen peroxide (Equation 6.12) or react with NO·, which is sometimes produced by these cells, to produce peroxynitrite (see Equation 6.10). Both products are bactericidal.

$$2\mathrm{O_2^{\bullet-}} + 2\mathrm{H^+} \rightarrow \mathrm{H_2O_2} + \mathrm{O_2} \tag{6.12}$$

Additionally, H_2O_2 in the presence of iron within the bacterium may produce cytotoxic ·OH via the Fenton reaction (see Equation 6.8). The enzyme myeloperoxidase occurs in neutrophils but not macrophages (Hampton et al., 1998). This enzyme, first isolated from human pus, is a nonspecific heme-containing peroxidase that in the presence of H_2O_2 catalyzes the oxidation of chloride ion (Cl^-) to hypochlorous acid (HOCl), a potent bactericidal oxidant.

Although the generation of ROS by these phagocytic cells is generally beneficial (to the host organism, anyway!), chronic inflammation can produce damage in local tissues and can contribute to diseases associated with oxidative stress, including arthritis and some cancers.

Antioxidant Defenses

As described above, the generation of ROS is an inescapable part of aerobic life. To flourish, all aerobic organisms have evolved a diverse array of mechanisms to minimize impacts due to ROS, and healthy organisms generally can cope well with the normal flux of ROS associated with respiration, certain enzyme activities, autooxidations, phagocytosis, and so forth. These mechanisms include enzyme systems that act to remove ROS, low-molecular-weight compounds that directly scavenge ROS (in animals, some produced endogenously and others obtained from the diet), and proteins that act to sequester prooxidants, particularly iron and copper. A number of these mechanisms, particularly some antioxidant enzymes, have been highly conserved in the course of evolution. Among vertebrates, most mechanisms are very similar, with differences among phyla generally being more quantitative than qualitative in nature; thus, extensive research in this area with mammalian models has greatly informed work with fishes. The following discussion of antioxidant defenses is condensed from studies with various models; a later section describes fish-specific studies.

Antioxidant Enzyme Systems

Superoxide Dismutases

A superoxide dismutase (SOD) was first isolated from bovine blood by McCord and Fridovich (1969); since that time, several forms of this enzyme have been identified and characterized (Fridovich, 1995). These enzymes accelerate the dismutation of $O_2^{\bullet-}$ by accepting an electron from one $O_2^{\bullet-}$ and passing it to another, yielding the following net reaction:

$$O_2^{\bullet-} + O_2^{\bullet-} \rightarrow H_2O_2 + O_2 \tag{6.13}$$

The observations that practically all aerobic organisms contain SOD, that many organisms including vertebrates produce several distinct SOD proteins, and that these enzymes enhance a reaction that occurs rapidly nonenzymatically support the theory that $O_2^{\bullet-}$ plays an important role in oxidative stress, despite its low reactivity vs. some other ROS (notably, ·OH).

Vertebrates contain three distinct forms of SOD: copper/zinc (CuZnSOD), manganese (MnSOD), and extracellular (ECSOD). CuZnSOD occurs predominantly in the cytosol, with some present in lysosomes and the nucleus. Eukaryotic CuZnSODs have a molecular mass of about 32,000 and contain two protein subunits, each containing one Cu and one Zn ion. At physiological pH, the uncatalyzed dismutation of $O_2^{\bullet-}$ has a rate constant on the order of 5×10^5 $M^{-1}s^{-1}$; bovine erythrocyte CuZnSOD accelerates this reaction to about 1.6×10^9 $M^{-1}s^{-1}$, effectively reducing the half-life of cellular $O_2^{\bullet-}$ by several orders of magnitude. CuZnSOD is highly sensitive to inhibition by cyanide, a characteristic useful in distinguishing its activity from MnSOD in analytical assays. Diethyldithiocarbamate is also a potent inhibitor of CuZnSOD.

Manganese SOD in animals is highly associated with mitochondria; it also occurs in bacteria and plants. Amino acid sequences of MnSODs across these phyla are quite similar (and distinct from those of CuZnSODs), consistent with the endosymbiotic theory that proposes that mitochondria evolved from a symbiosis between an early CuZnSOD-containing eukaryote and a MnSOD-containing prokaryote

(Fridovich, 1995). Interestingly, the blue crab (*Callinectes sapidus*) and other marine crustaceans that rely on Cu-dependent hemocyanins for O_2 transport contain no CuZnSOD, but have MnSOD in both mitochondria and cytosol (Brouwer et al., 1997). Vertebrate MnSODs have a molecular mass of about 92,000, and they usually contain four protein subunits and 2 or 4 Mn ions per enzyme. MnSODs are insensitive to inhibition by both cyanide and diethyldithiocarbamate. Despite these differences between MnSOD and CuZnSOD, both catalyze essentially the same reaction (Equation 6.13).

Extracellular SOD is also a CuZnSOD present in mammals (unknown for other vertebrates), but it has a far higher molecular weight than cytosolic CuZnSOD (135,000 vs. 32,000). ECSOD is a tetrameric glycoprotein in which each subunit contains one Cu and one Zn ion. It is particularly abundant in the extracellular space of blood vessels, bound to heparin sulfate, the glycosaminoglycan component of heparin sulfate proteoglycans that interact with various proteins on cell surfaces (Fukai et al., 2002). The large mass of this tetrameric protein and its affinity for cell surfaces prevents filtration by the kidneys (Fridovich, 1998). In blood vessels, ECSOD appears to play a critical role in protecting $NO\cdot$ from $O_2^{\bullet-}$. $NO\cdot$ produced by endothelial cells stimulates smooth muscle relaxation; thus, diminished ECSOD expression or activity may play a role in cardiovascular diseases (Fukai et al., 2002).

Another SOD found in bacteria, algae, and higher plants (but not observed in animal tissues) contains iron; hence, it is termed FeSOD. Most FeSODs contain two subunits, and each enzyme molecule has one or two iron ions. The bacterium *Escherichia coli* also contains a hybrid dimeric SOD, with one subunit from MnSOD and the other from FeSOD; this hybridization is facilitated by the similar amino acid sequences shared by the two forms (Fridovich, 1995).

Catalases

Although SODs very effectively reduce cellular $O_2^{\bullet-}$ concentrations, the downside of this activity is that one of the two products produced is H_2O_2. Hydrogen peroxide can be damaging in its own right, and in the presence of transition metals such as copper and iron it can also serve as the precursor to highly reactive $\cdot OH$. Most aerobic organisms and all vertebrates possess two enzyme systems that metabolize H_2O_2: the catalases and peroxidases (including glutathione peroxidases found in vertebrates, discussed below). Peroxidases generally can act on a variety of organic peroxides as well as H_2O_2, whereas catalases are largely restricted to H_2O_2.

Vertebrate catalases are large proteins with a molecular mass of 120,000 and consisting of four subunits, each containing a ferric heme group at its active site (Reid et al., 1981). The heme groups are deep within the subunits and accessed by narrow channels, which accounts for the substrate specificity of catalase. They catalyze a reaction conceptually similar to that catalyzed by SOD, a dismutation reaction (Equation 6.14). Aminotriazole is an effective catalase inhibitor (Darr and Fridovich, 1986).

$$2H_2O_2 \rightarrow O_2 + 2H_2O \tag{6.14}$$

The major cellular location of vertebrate catalase is in an organelle known as the *peroxisome*. Peroxisomes function in the β-oxidation of fatty acids, and H_2O_2 is produced as a byproduct of this process; thus, catalase prevents damage to peroxisomes and also impedes the movement of H_2O_2 to other locations in the cell (due to its uncharged nature, H_2O_2 traverses organelle membranes more readily than other ROS). The role that catalases play in the metabolism of H_2O_2 produced outside of peroxisomes appears to vary among tissues. The next enzyme discussed, glutathione peroxidase, has a broader distribution within cells and likely plays a more important role in clearing hydrogen (and other) peroxides produced in some tissues, such as liver; however, under conditions elevating H_2O_2 production in some tissues including erythrocytes (that do not contain peroxisomes), lung, and eye, catalase activity may be particularly important (Halliwell and Gutteridge, 1999).

Glutathione Peroxidases and Transferases

Glutathione peroxidases (GPXs) provide another mechanism by which animals can detoxify H_2O_2 (Arthur, 2000); moreover, they can also reduce fatty acid peroxides (LOOH), an important manifestation of oxidative stress described later. Four selenium-dependent GPXs have been identified in mammals:

classical GPX (GPX1), gastrointestinal GPX (GPX2), plasma GPX (GPX3), and phospholipid hydroperoxide GPX (GPX4 or PHGPX). All contain selenocysteine at the active site. Selenocysteine is an amino acid analogous to cysteine in which the sulfur atom has been replaced by selenium. It is inserted by a specific tRNA into selenoproteins, including GPXs. Most GPXs characterized in vertebrates contain four subunits, each containing one selenium residue; PHGPX, however, is monomeric.

Little work has been done verifying the nature of GPXs in fish, although based on tissues examined and substrates used, it is likely that most selenium-dependent GPX activity measured in fish thus far corresponds to GPX1. Kryukov and Gladyshev (2000) detected 18 genes in zebrafish that contained selenocysteine. Two appeared very similar to one another and resembled both human GPX1 and GPX2; the other two were also very similar to one another and resembled human GPX4. The occurrence of highly matched pairs probably arose from a gene duplication event in many fishes, including zebrafish.

The reactions catalyzed by GPXs involve the reduction of a peroxide substrate to its corresponding alcohol, coupled with the oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG) (Chance et al., 1979). In the case of H_2O_2, the corresponding alcohol is water (Equation 6.15); with lipid peroxides, it is the corresponding lipid alcohol (LOH, Equation 6.16):

$$H_2O_2 + 2GSH \rightarrow GSSG + H_2O \tag{6.15}$$

$$LOOH \rightarrow 2GSH \rightarrow GSSG + LOH \tag{6.16}$$

An important distinction between catalase and GPX is the ability of only the latter to reduce lipid peroxides; however, most GPXs, including classical GPX, cannot metabolize lipid peroxides that are esterified to lipid molecules in membranes, for example. Such peroxides must first be released by lipase activity. An exception to this is PHGPX, which can directly reduce lipid peroxides associated with membranes (and also free LOOH), as well as thymine hydroperoxide, a form of oxidative DNA damage (Bao and Williamson, 2000).

Some glutathione *S*-transferases (GSTs; extensively discussed in Chapter 4) exhibit peroxidase activity, which is specific for LOOH (they are unable to act on H_2O_2). This activity is sometimes referred to as *selenium-independent* or *non-selenium* GPX activity, as GSTs contain no selenium. Although it can account for an appreciable portion of total GPX activity measured in tissue preparations with standard substrates such as cumene hydroperoxide, its significance *in vivo* is unclear (Halliwell and Gutteridge, 1999). Quantification of real GPX activity using H_2O_2 as substrate is sometimes preferred. Some GSTs play another role in antioxidant defense by conjugating breakdown products from lipid peroxidation such as 4-hydroxynonenal (Hayes et al., 2005).

Glutathione: Synthesis and Maintenance

As should be clear from earlier discussions of GSTs (Chapter 4) and discussions in this chapter, glutathione (GSH) plays critical roles in the protection of cells from chemical insult. It is also plays additional roles in metabolism, biosynthesis, transport, and cellular communication; thus, a brief description of this molecule with particular reference to oxidative stress is warranted at this juncture. GSH is the tripeptide γ-glutamyl-cysteinyl-glycine (Figure 6.1); concentrations vary widely among species and tissue but are generally far higher in mammals than amino acid concentrations, typically occurring in the low millimolar range (Griffith and Mulcahy, 1999); similar levels have been reported in fishes (see below). GSH is synthesized through two reactions, the first catalyzed by glutamate cysteine ligase (GCL; previously termed γ-glutamylcysteine synthase, or GCS) and the second by GSH synthetase (Equation 6.17 and Equation 6.18, respectively):

$$\text{L-glutamate} + \text{L-cysteine} + ATP \rightarrow \text{L-}\gamma\text{-glutamylcysteine} + ADP + P_i \tag{6.17}$$

$$\text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{L-glycine} + ATP \rightarrow GSH + ADP + P_i \tag{6.18}$$

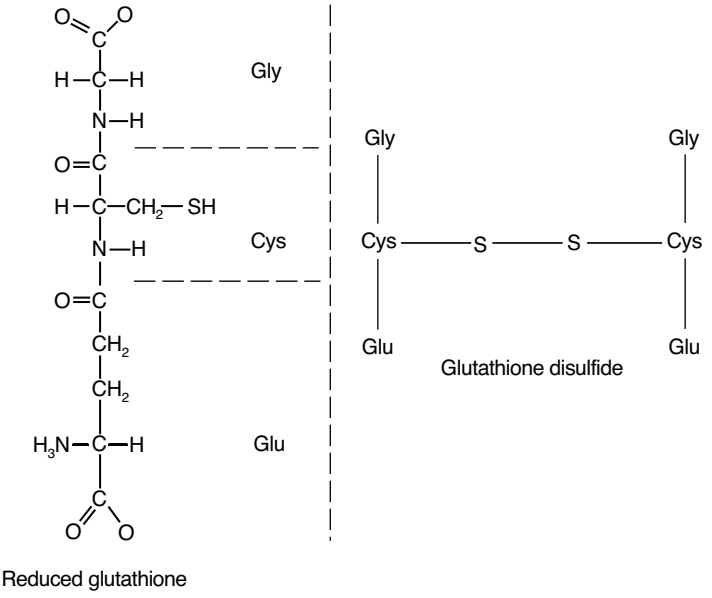

FIGURE 6.1 Structures of reduced glutathione (GSH) and glutathione disulfide (GSSG).

Glutamate cysteine ligase is the rate-limiting enzyme of GSH synthesis; it is sensitive to inhibition by buthionine sulfoximine (BSO), which can be used experimentally to deplete cellular GSH (Meister, 1995). GCL has been purified from a number of eukaryotes, and all are heterodimers composed of a heavy and a light subunit (molecular weights of about 73,000 and 31,000, respectively) (Griffith, 1999; Griffith and Mulcahy, 1999). The heavy subunit (GCL_h or GCLC, where C stands for "catalytic") is responsible for catalytic activity; the light subunit (GCL_l or GCLM, where M stands for "modifier") plays a regulatory function. In mammals, genes for the two are on separate chromosomes, and their transcription does not appear to be highly coordinated, although both have an antioxidant response element (ARE) in the promoter region (discussed below). Factors affecting GCL activity include availability of substrates (glutamate and cysteine) and feedback inhibition by GSH. GCLM modulates activity of the complete enzyme essentially by reducing K_m values for substrate concentrations and increasing the K_i for GSH.

As described above, GPX couples the reduction of H_2O_2 and LOOH to corresponding alcohols with the oxidation of GSH to GSSG. GSH can also directly scavenge ROS, including $O_2^{\bullet-}$, ·OH, RO·, ROO·, and $ONOO^-$ (Griffith, 1999; Halliwell and Gutteridge, 1999), with the thiol (–SH) group provided by cysteine as the active moiety that undergoes oxidation. In these cases as well, GSSG arises from GSH oxidation as a consequence. GSSG contains the backbones of two GSH molecules, covalently linked through a disulfide bond (Figure 6.1). Normal healthy cells not experiencing undue oxidative pressure typically exhibit GSH:GSSG ratios approaching or greater than 100:1, and declines in this ratio have been used as a marker of oxidative stress. This glutathione redox balance is maintained by GSH synthesis, export of oxidized and conjugated glutathione, and activity of glutathione reductase (GR), which catalyzes the reduction of GSSG to GSH:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \tag{6.19}$$

Glutathione reductase contains two subunits, each with fatty acid desaturase (FAD) at its active site (Thienne et al., 1981). Electrons provided by NADPH (produced by the pentose phosphate pathway) are apparently passed through FAD en route to their reduction of GSSG. In addition to the direct effects of ROS described below, the energetic costs of antioxidant defense activity are significant, as illustrated by the energy required to produce, maintain, and utilize GSH for this role.

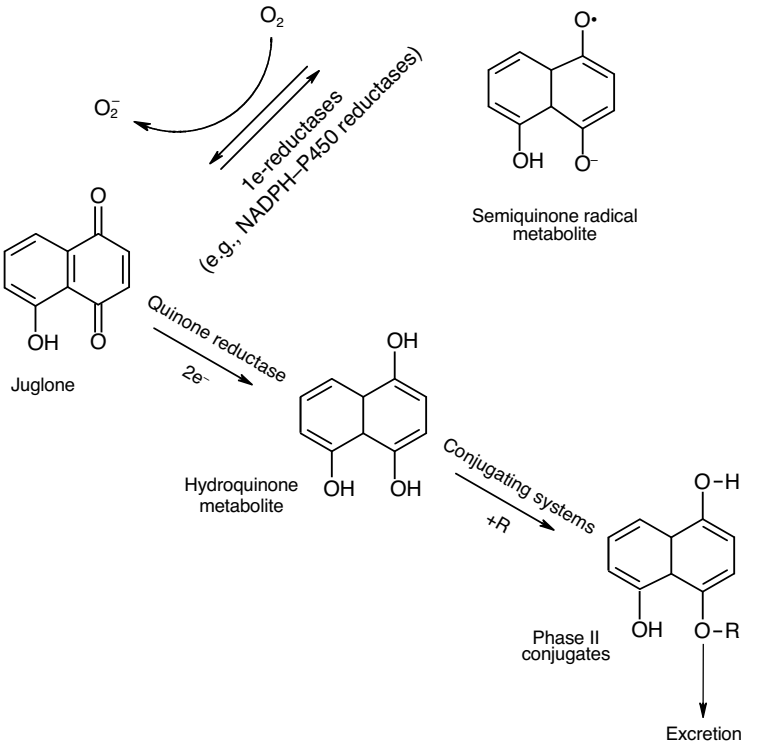

FIGURE 6.2 Metabolism of the plant-derived quinone juglone. Upper right portion depicts redox cycling through the semiquinone radical intermediate, a one-electron reduction product. The lower pathway depicts the two electron reduction pathway, which can be catalyzed by DT diaphorase (NAD(P)H:quinone oxidoreductase). This pathway yields phenolic compounds that are readily detoxified by phase II pathways (see Chapter 4).

Quinone Reductases

Quinone reductases do not directly act on ROS; however, by reducing quinones they can diminish ROS that are generated by quinones during the process of redox cycling, described below. Because quinones comprise an important group of environmental contaminants (as well as natural products), the inclusion of quinone reductase here is warranted (although it is also grouped with phase II enzymes, described in Chapter 4). Quinones, such as the natural product juglone (Figure 6.2), can exert toxicity via two mechanisms: reactions with –SH groups of GSH and proteins, leading to GSH depletion and enzyme inactivation, for example, and through generation of ROS via redox cycling (Bolton et al., 2000; Dinkova-Kostova and Talalay, 2000).

In the process of redox cycling, quinones can gain an electron from a reduced source, such as NADPH–cytochrome P450 reductase (see Chapter 4), yielding a semiquinone radical (Figure 6.2). Under aerobic conditions, this radical can donate its unshared electron to O_2, producing $O_2^{\bullet-}$. Quinone reductases circumvent this process by catalyzing a two-electron reduction of the quinone to its corresponding hydroquinone. In addition to quenching ROS formation, the hydroquinone products thus formed are oftentimes excellent substrates for phase II enzymes, particularly sulfotransferases (ST) and UDP-glucuronosyltransferase (UGT) (see Chapter 4); thus, quinone reductase activity also enhances elimination.

In mammals, two distinct quinone reductases have been identified; both are primarily cytosolic and both contain FAD (Foster et al., 2000). Until recently, only one had been recognized; it is formally termed NAD(P)H:quinone oxidoreductase and is also referred to as DT diaphorase. Zhao et al (1997), through comparisons with a cDNA clone isolated by Jaiswal (1994a), demonstrated that a previously ignored protein with quinone reductase activity (described by Liao and Williams-Ashman, 1961) had close sequence homology to DT diaphorase. This "rediscovered" protein structurally resembles a truncated version of DT diaphorase but is a separate gene product. These enzymes are now oftentimes

referred to as *quinone reductase type 1* (QR1), which is DT diaphorase, and *quinone reductase type 2* (QR2). Major differences between QR1 and QR2 include functional electron donors (QR1 employs NADH and NADPH, but QR2 employs nonphosphorylated nicotinamides), inhibition (QR1 but not QR2 is very sensitive to dicoumarol), and regulation. QR1 is highly inducible through both xenobiotic response elements and antioxidant response elements (described below), but QR2 is apparently not inducible. Fish clearly express QR1 (see below), but we are unaware of reports concerning QR2 in this vertebrate class.

The proteins discussed here are generally considered major players as antioxidant enzymes; however, they do not include all enzymes known to have some antioxidant function. Peroxiredoxin (Georgiou and Masip, 2003), thioredoxin and glutaredoxin (Holmgren, 2000; Watson et al., 2004), heme oxygenase (Kvam et al., 1999), and other enzymes may play important roles at well, at least under some circumstances. For additional information, see Halliwell and Gutteridge (1999).

Low-Molecular-Weight Antioxidants

A number of biomolecules can directly scavenge ROS nonenzymatically. GSH, discussed above, is a very important component of this group, although it is also essential to GPX activity. Several other prominent antioxidants are vitamin related and are obtained through the diet by most animals. These include ascorbic acid (vitamin C), tocopherols (vitamin E components), and carotenoids (vitamin A, or retinol, precursors). A number of other compounds synthesized by animals exhibit antioxidant capacity *in vitro*, but their antioxidant functions *in vivo* generally remain unclear.

Ascorbic Acid (Vitamin C)

Plants and many animals can synthesize ascorbic acid from glucose, although humans and other primates, bats, and passerine birds cannot (Moreau and Dabrowski, 2003; Nishikimi and Yagi, 1996). Among fishes, teleosts cannot perform this synthesis, but those retaining more ancestral characteristics such as lampreys, sharks, rays, lungfishes, sturgeons, paddlefishes, and bowfin can (Moreau and Dabrowski, 1998). Those animals that cannot synthesize ascorbic acid lack the enzyme that catalyzes the terminal step in biosynthesis, gulonolactone oxidase. A DNA sequence resembling the gene for this enzyme in other animals has been identified in humans and guinea pigs, but it is extensively mutated and inactive (Nishikimi and Yagi, 1996).

At physiological pH, ascorbic acid exists largely as ascorbate (Figure 6.3) and is highly water soluble. Ascorbate serves as a cofactor for a number of enzymes, including proline hydroxylase and lysine hydroxylase, that are involved in collagen synthesis, as well as for dopamine-β-hydroxylase, which catalyzes the conversion of dopamine into norepinephrine (Nishikimi and Yagi, 1996). The hallmark human disease associated with vitamin C deficiency is scurvy, a common disease among sailors in earlier times (before the importance of fresh fruit was recognized); it is characterized by muscle weakness, blood vessel fragility, and bleeding from gums and other mucous membranes.

Ascorbate is a powerful reducing agent and has been shown with numerous *in vitro* studies to scavenge a number of ROS, including $O_2^{\bullet-}$, ·OH, ROO·, and HOCl (Halliwell, 1996). Although unequivocally proving the significance of these reactions *in vivo* is difficult, the function of ascorbate as an antioxidant is widely accepted based on considerations of its *in vitro* reactivity, concentrations in tissues, experimental manipulations of its levels in animals and accompanying markers of oxidative stress, and related consequences in human deficiencies (Halliwell and Gutteridge, 1999).

Upon reduction of an oxygen radical, ascorbate is oxidized to the ascorbyl radical (also known as semidehydroascorbate) (Bendich et al., 1986). This radical is relatively stable and unreactive, a feature central to its antioxidant function; that is, a much more reactive free radical reacts with ascorbate, yielding a less reactive and hence more innocuous ascorbyl radical. The ascorbyl radical is readily oxidized again, yielding dehydroascorbate (DHA, the two-electron oxidation metabolite of ascorbate). DHA has little antioxidant activity and can break down to several products (notably oxalic acid and threonic acid) or be reduced back to ascorbate by an apparently nonspecific GSH-dependent reductase (Wells et al., 1990).

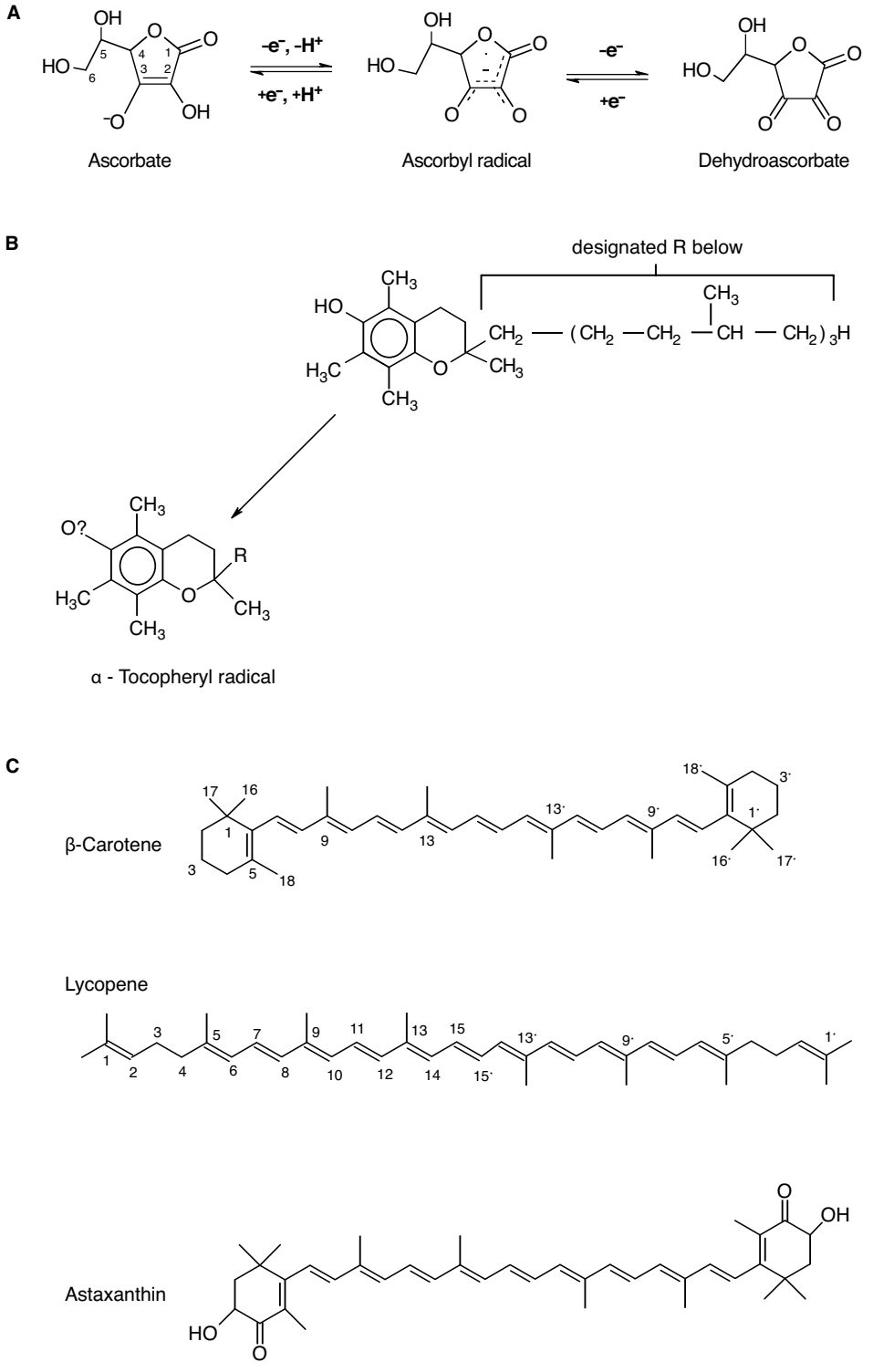

FIGURE 6.3 Low-molecular-weight antioxidants. (A) Ascorbate (vitamin C) and its one-electron (ascorbyl radical) and two-electron (dehydroascorbate) oxidation products. (B) α-Tocopherol (vitamin E) and its radical product. (C) Representative carotenoids.

Vitamin E

Vitamin E is a highly effective lipid soluble scavenger of peroxyl radicals and hence a key protectant against membrane damage via lipid peroxidation (Niki and Matsuo, 1993). It appears to be a dietary requirement of all animals. Vitamin E is actually a mixture of several tocopherols and tocotrienols; the major component acting in animal cells is α-tocopherol (Figure 6.3) (Diplock, 1985). α-Tocopherol (in addition to other tocopherols and tocotrienols) is effective at protecting membranes from oxidative attack by lipid peroxyl radicals ($LOO^{\cdot}$) because it is more reactive with these radicals than are membrane lipids and proteins. Vitamin E components are also effective scavengers of $O_2^{\cdot -}$, $^{\cdot}OH$, and $^1O_2$. As with ascorbate, α-tocopherol itself becomes a radical species upon reacting with an oxygen radical (Figure 6.3). α-Tocopherol can be regenerated from its radical form by mechanisms including reduction of the tocopherol radical by ascorbate; this reaction appears to represent an important cooperation between these vitamins to maintain active α-tocopherol within membranes (Traber, 1994).

Carotenoids

Carotenoids are a large group (over 600 described) of plant pigments, some of which are absorbed from the diet in many animals (Krinsky, 1993). Carotenoid structure generally includes about 40 carbons, most of which occur in alternating single and double bonds, a feature that allows for electron delocalization and absorbance of light in the visible range (Britton, 1995). Either or both ends of this carbon chain are often modified into cyclic rings, which may also contain oxygen groups; several carotenoids are illustrated in Figure 6.3. Carotenoids are very lipophilic and virtually insoluble in water. Many, including the most abundant carotenoid observed in humans, β-carotene (Figure 6.3), serve as precursors for vitamin A (retinol) and retinoic acid, which play important roles in cell growth, differentiation, and development. Some, such as astaxanthin (Figure 6.3), contribute to the red coloration of some fishes, including male sticklebacks, for which this coloration plays a role in sexual preference by female sticklebacks (Barber et al., 2000).

In terms of antioxidant function, the best established role for carotenoids is that of a scavenger of singlet oxygen in plant chloroplasts (Telfer et al., 1994); $^1O_2$ is generated at high rates during photosynthesis. The ability of carotenoids to scavenge a variety of oxygen radicals has been demonstrated *in vitro* (Liebler and McClure, 1996). Although it is generally believed that they also a serve a significant antioxidant function in animals *in vivo*, this has not been firmly established.

Other Antioxidants

A number of endogenous compounds with various well-characterized functions other than acting as an antioxidant have been shown to be also capable of scavenging ROS in various *in vitro* systems. These include bilirubin, estradiol, lipoic acid, coenzyme Q (ubiquinol), and uric acid (Halliwell and Gutteridge, 1999); however, the significance of *in vitro* studies suggesting an antioxidant function to the *in vivo* situation is oftentimes difficult to gauge.

Another potentially important function of some compounds, other than direct ROS scavenging, is regulation of metals that catalyze ROS production (via Fenton-like chemistry, for example). Iron and copper are the two essential transition metals that naturally occur in vertebrates at relatively high concentrations, and if they are free in cells they can readily enhance ROS generation, lipid peroxidation, and other manifestations of oxidative stress. Consequently, cells have mechanisms to carefully regulate them, keeping them available for incorporation into appropriate metalloproteins, for example, while preventing them from participating in destructive oxidative reactions, such as Fenton reactions and autooxidations.

For iron, perhaps the most important regulatory protein is ferritin (Harrison and Arosio, 1996; Orino et al., 2001). Most intracellular iron is bound to ferritin, which forms a large shell-like structure comprised in mammals of 24 subunits each of about 20,000 molecular weight and distinguished into two types: H and L. Up to 4500 iron ions can be stored in the core of a protein. H subunits are involved in iron detoxification by virtue of ferroxidase activity that oxidizes Fe^{2+} to Fe^{3+}. L subunits facilitate nucleation of Fe^{3+} within the core of ferritin for long-term storage. A similarly structured ferritin has been purified from the marine fish *Dasyatis akajei* (Kong et al., 2003).

Ceruloplasmin serves an analogous function for copper (Harris, 1995). Dietary copper entering the bloodstream largely binds to albumin; this complex is taken up by the liver, where the copper is incorporated into ceruloplasmin. Ceruloplasmin has a molecular weight of about 132,000 and tightly binds six copper ions. The copper–ceruloplasmin complex is secreted by the liver into the plasma and can contribute copper to cells requiring it following binding to cell surface receptors. Ceruloplasmin has been found in several fish species, including carp, plaice, mullet, tilapia, and European eel (Grosell et al., 1998).

Metallothioneins (MTs) are low-molecular-weight (about 6500) proteins, the primary role of which is unclear, although evidence suggests that antioxidant activity may be at least an ancillary function (Coyle et al., 2002). Most research has focused on their roles in metal metabolism and homeostasis, including metal detoxification (Klaassen et al., 1999). They are very cysteine rich (about 30% of amino acid residues), which underlies their high binding affinities for a number of metals, including copper, cadmium, zinc, silver, bismuth, and mercury. Two isoforms, MT-1 and MT-2, appear to occur in all animal tissues and are the most-studied MTs. They typically bind five to seven metal ions per molecule and are inducible by some metals (including those mentioned above), stress hormones (such as glucocorticoids), oxidants, and inflammation. Considerable debate currently exists regarding whether their primary role is regulation of essential metals, particularly copper and zinc, for use in metalloenzymes vs. protection against metal toxicity, particularly from cadmium. Additionally, several lines of experimental evidence support an antioxidant function for MTs, including inducibility by prooxidants such as hydrogen peroxide and paraquat, protection against such chemicals in MT-enriched vs. MT-depleted cells (via transfections and gene knock-out models, for example), and the ability of MT to scavenge ROS and spare GSH (Klaassen et al., 1999). Whether or not these results have significant *in vivo* ramifications remains unclear.

Reactive Oxygen Species and Gene Expression

Antioxidant defenses can be altered in response to exposure to oxidative stress in a variety of ways, and the mechanisms by which such regulation occurs have been extensively studied in prokaryotic and mammalian systems. Expression and activity of antioxidant, as well as prooxidant, gene products are up- or downregulated at the levels of mRNA transcription, mRNA stabilization, and protein activation; however, the mechanisms by which these alterations occur, as well as the genes involved, are much more complex in eukaryotes than in prokaryotes.

ROS-Mediated Modulation of Gene Expression in Prokaryotes

In prokaryotes, well-defined redox-sensitive transcription factors first recognize ROS and then activate the transcription of antioxidant genes (Bauer et al., 1999). For example, the OxyR protein is specifically oxidized by (recognizes) hydrogen peroxide; the oxidized form of the protein, in which a disulfide bond has been created, drives transcription of genes, including MnSOD, catalase, glutathione reductase, hydroperoxidase, heat shock proteins, and glutaredoxin (Bauer et al., 1999; Zheng and Storz, 2000). Similarly, a [2Fe–2S] cluster in the SoxR protein is oxidized in the presence of superoxide-generating chemicals, at which point SoxR drives the transcription of the *soxS* gene. The SoxS protein, a transcription factor, acts to increase expression of genes including MnSOD, glucose-6-phosphate dehydrogenase, NADPH:flavodoxin oxidoreductase, aconitase, and endonuclease IV (Zheng and Storz, 2000). Interestingly, in some cases the proteins induced play an indirect rather than direct role in conferring resistance to oxidative stress. For example, fumarase C is induced via SoxS under conditions of oxidative stress, although fumarase, a citric acid cycle enzyme, is not itself an antioxidant. Fumarase C induction is essential under conditions of oxidative stress because the other forms of this enzyme, fumarases A and B, are inactivated by high oxygen conditions (Liochev and Fridovich, 1992).

Overview of ROS-Mediated Modulation of Gene Expression in Eukaryotes

Although an understanding of ROS-driven gene regulation in prokaryotes can be useful in developing hypotheses regarding responses to ROS in eukaryotic organisms, significant differences exist between the prokaryotes and eukaryotes thus far studied (Crawford, 1999; McCord, 2000). One major difference is that transcription factors that are responsive exclusively to ROS have not been identified in eukaryotes; rather, signaling molecules also involved in responses to other stimuli are used. Another major difference is that a different array of proteins is induced in eukaryotes, including many that are not thought of as classical antioxidants and sometimes not including proteins that typically are thought of as classical antioxidants. A third major difference is the presence in some mammalian species of an enhancer element known as the antioxidant response element (ARE), which regulates the expression of certain antioxidants and other proteins. Some of these differences may be attributable to the large differences that exist between single-cell and multicellular organisms in terms of their dependence on oxygen and the desirability of maximizing rates of cell division (McCord, 2000). These differences are discussed at greater length below.

The expression of many genes is affected by ROS; however, it is important to bear in mind that not all such changes necessarily represent adaptive, antioxidant responses. Some changes in gene expression simply result from oxidative damage; for example, low levels of damage may alter the activity of transcription factors (Gutteridge and Halliwell, 1999). Additionally, ROS are generated purposefully not only as physiological effector molecules (such as $O_2^{\bullet-}$ generated by phagocytes, discussed previously) but also as signaling molecules in pathways not necessarily related to oxidative stress *per se* (Finkel, 2000; Morel and Barouki, 1999; Palmer and Paulson, 1997; Sauer et al., 2001). Thus, ROS may be generated by enzymes such as NADPH oxidase or in the mitochondria as part of normally functioning signaling pathways (Palmer and Paulson, 1997; Sauer et al., 2001). It may be useful to conceptualize the effect of the signaling elicited by ROS as varying qualitatively according to the amount and duration of the associated oxidative stress, with low levels in some cases producing a cellular response of proliferation at least *in vitro*, intermediate levels often producing temporary growth arrest and an adaptive response, higher levels leading to apoptosis, and very high doses causing necrosis (Chandra et al., 2000; Davies, 1999; Gutteridge and Halliwell, 1999; Martindale and Holbrook, 2002). Thus, ROS cannot be thought of as simply and exclusively damage-causing agents; rather, it is the production of inappropriate amounts of ROS or production in the wrong place or at the wrong time that is problematic. Furthermore, the ability of ROS-generating chemicals to cause damage is not limited to their ability to generate highly reactive toxic intermediates but is also related to their ability to alter the functioning of normal signaling pathways that utilize ROS as messenger molecules. Finally, signaling by reactive nitrogen, copper, iron, and other redox-active metal species, in addition to reactive oxygen species, likely plays an important role in modulating gene expression (Gutteridge and Halliwell, 1999).

Effects of ROS on Transcription Factors

The activity of many transcription factors is dependent upon the reduced or oxidized state of redox-responsive moieties such as thiols or Fe–S clusters; as a result, oxidative stress can alter the activity of those transcription factors by altering the redox status of the cell (Arrigo, 1999; Primiano et al., 1997; Schafer and Butner, 2001; Sen, 2000). Transcription factors in eukaryotes reported to be affected in this fashion include AP-1, Maf, Nrl, NF-IL6, Sp-1 family members, protein kinase C, glucocorticoid receptors, estrogen receptors, aryl hydrocarbon receptor, NF-κB, p53, and others (Arrigo, 1999; Crawford, 1999; Dalton et al., 1999; Michiels et al., 2002; Primiano et al., 1997; Sen, 2000). ROS also increase the rate of transcription of many transcription factors, including members of the AP-1, NF-κB, and AP-2 families (Dalton et al., 1999). The upstream events leading to activation of transcription of these factors are complex and include alterations in phosphorylation of signaling molecules, release of arachidonic acid from cell membranes and subsequent metabolism of the arachidonic acid, and mobilization of Ca^{2+} (for reviews, see Dalton et al., 1999; Martindale and Holbrook, 2002; Sauer et al., 2001).

Effects of ROS on mRNA Expression

The transcription factors mentioned above are involved in numerous signaling pathways, including pathways related to cell division and differentiation, immunological response, cytokine expression and inflammatory response, xenobiotic metabolism, and many others (Finkel and Holbrook, 2000; Martindale and Holbrook, 2002; Sauer et al., 2001); thus, it is not surprising that exposure to high levels of ROS induces the expression of many genes. A small sampling of the many genes reported to be regulated (in at least some cases) in eukaryotes by ROS include c-*fos* and c-*myc* (cellular protooncogenes), *gadd*45 and *gadd*153 (growth arrest and DNA damage genes), γ-glutamyl transpeptidase, GCL (both subunits), some GST isoforms, some UGT isoforms, epoxide hydrolase, heme oxygenase, MnSOD and ECSOD but not usually CuZnSOD, metallothionein, GPX1, interleukin 8, cytochrome IV, thioredoxin, and an aldo-keto reductase (Brady et al., 1997; Burczynski et al., 1999; Crawford, 1999; Dhakshinamoorthy et al., 2000; Prestera et al., 1993; Schull et al., 1991). The expression of some of these (and other) genes is regulated by ROS via the antioxidant response element, as discussed below. As indicated by this partial listing, although many of the genes identified as upregulated by ROS could clearly play important antioxidant roles, many others are not classical antioxidants. Furthermore, the degree of induction observed in the classical antioxidants is often relatively low; heme oxygenase, not a classical antioxidant, appears to be one of the most responsive markers of oxidative stress in cells, as it shows a high degree of induction as well as specificity for oxidative stress as an inducer (Crawford, 1999). Further confusing the picture are observations that, in some cases, classical antioxidants are upregulated to a greater degree by inducers other than typical oxidants; for example, MnSOD is upregulated 20- to 100-fold by cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), compared to a usually less than 5-fold induction by prooxidants such as xanthine oxidase, paraquat, iron, copper, and *t*-butyl hydroperoxide (Crawford, 1999; Stralin and Marklund, 1994; Valentine and Nick, 1999; Visner et al., 1990).

The expression of many genes is also downregulated by oxidative stress; among these are many mitochondrial genes (Crawford 1999; Fujii and Taniguchi, 1999; Morel and Barouki, 1999) and a large number of nuclear-encoded genes involved in the immune response, cell replication, carbohydrate metabolism, hormonal responses, phase I xenobiotic metabolism, and other cellular activities (Barouki and Morel, 2001; Morel and Barouki, 1999; Nebert et al., 2000). As in the case of ROS-mediated gene induction, these results seem likely to reflect not only adaptive changes directed at decreasing oxidative damage but also alterations in pathways in which ROS normally play a role as signaling molecules or simple oxidative damage to cellular macromolecules that mediate gene expression in these pathways.

Antioxidant Response Element (ARE)-Mediated Gene Regulation

The only known molecular mechanism for specifically responding to high levels of ROS in eukaryotes is the activation of a relatively large number of genes via an enhancer element termed the antioxidant response element (ARE), or electrophile response element (Dalton et al., 1999; Itoh et al., 1999; Nguyen et al., 2003b; Rushmore et al., 1991; Wasserman and Fahl, 1997). Many genes have been shown to be regulated by AREs, and many others are suspected to be ARE regulated, based on the presence of ARE sequences in the promoter regions of those genes, the inducibility of those genes by chemicals that either generate or scavenge ROS, and the regulation of those genes by putative ARE transcription factors such as Nrf1 and Nrf2 (discussed below). A list of genes known or suspected to be regulated by the ARE, grouped according to the type of evidence currently available in the literature, is presented in Table 6.1.

The identity and role of all of the transcription factors that bind to the ARE are not yet clearly defined but are an area of very active investigation. Early suggestions that AP-1 was involved turned out to be inaccurate, except in those cases where the ARE is part of a functional 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) (Dalton et al., 1999), such as the case of the human QR1 gene (Jaiswal 1994b). Thus, although the ARE bears strong sequence similarity to the TRE, they are not the same. Furthermore, although there are similarities in terms of the transcription factors that bind the two sites, they are not identical, and the combinations that bind are usually different. At this point, the best evidence suggests that ARE transcription factors include one or more CNC-bZIP proteins (e.g., Nrf1 and especially Nrf2), perhaps as part of a heterodimer with a small

TABLE 6.1

Genes Regulated or Possibly Regulated by the Antioxidant Response Element (ARE) in Mammals

Evidence for ARE Regulation	Gene	Ref.
Functional ARE characterized	Rat QR1	Favreau and Pickett (1991)
	Human QR1	Li and Jaiswal (1992)
	Human QR2	Jaiswal (1994a)
	Rat GST-Ya	Rushmore et al. (1991)
	Mouse GST-Ya	Friling et al. (1990)
	Rat GSTP	Okuda et al. (1989)
	Mouse ferritin L	Wasserman and Fahl (1997)
	Mouse ferritin H	Tsuji et al. (2000
	Human GCLC	Mulcahy et al. (1997)
	Human GCLR	Moinova and Mulcahy (1998)
	Mouse heme oxygenase	Alam et al. (1995)
	Human c-*jun*	Radjendirane and Jaiswal (1999)
	Mouse metallothionein 1	Dalton et al. (1994)
	Mouse cystine/glutamate exchange transporter	Sasaki et al. (2002)
ARE sequence identified but functionality not demonstrated	Human multidrug resistance protein 1	Kurz et al. (2001)
	Mouse UDPGT1A6	Vasiliou et al. (1995)
	Mouse aldehyde dehydrogenase 3A1	Vasiliou et al. (1995)
	Rat aflatoxin B_1 aldehyde reductase AKR7A1	Ellis et al. (2003)
	Human P450 aromatase	Wasserman and Fahl (1997)
	Human myoglobin	Wasserman and Fahl (1997)
	Human β-globin	Wasserman and Fahl (1997)
	Human collagenase	Wasserman and Fahl (1997)
	Mouse MnSOD	Jones et al. (1995)
Upregulation of gene by antioxidants or ROS-generating chemicals	Rat P4502B1 and 2B2; GSTYc1 and Yc2	Buetler et al. (1995)
	Human dihydrodiol dehydrogenase	Burczynski et al. (1999)
	Rat microsomal EH	Lamb and Franklin (2000)
	Human UDPGT1A9	Münzel et al. (1999)
	Mouse aldehyde dehydrogenase 3A1	Sládek (2003)
	Multidrug resistance protein 2	Bock et al. (2000)
	Human homolog of Keap-1, thioredoxin reductase, GR	Li et al. (2002)
	Hamster GPx1	Schull et al. (1991)
Regulation of basal or inducible expression of gene by Nrf1 or Nrf2	Glutathione synthetase	Kwong et al. (1999)
	Mouse microsomal epoxide hydrolase, aflatoxin B_1 aldehyde reductase, MnSOD, catalase, GST Yp, GST Yc	Kwak et al. (2001)

Note: The genes are sorted according to the type of evidence indicating that they are or may be ARE regulated.

Maf protein or c-Jun (Dalton et al., 1999; Dinkova-Kostova et al., 2002; Itoh et al., 1999; Jeyapaul and Jaiswal, 2000; Kwong et al., 1999; Nguyen et al., 2003b; Zipper and Mulcahy, 2002), and possibly other proteins as well (Nguyen et al., 2003b; Wasserman and Fahl, 1997). Recent evidence suggests that the "redox sensor" that activates this pathway is the cytoplasmic Nrf2-binding protein Keap1; oxidation of sulfhydryl groups in Keap1 leads to the release of Nrf2, permitting it to move to the nucleus, heterodimerize, and act as a transcription factor (Dinkova-Kostova et al., 2002). Phosphorylation of Nrf2 also appears likely to regulate the transcriptional activity of Nrf2 (Huang et al., 2000; Kong et al., 2001), perhaps in part via an increase in Nrf2 stability (Nguyen et al., 2003a). Electrophile-induced polyubiquitination of the Keap1 protein may play a role in Nrf2 release as well (Eggler et al., 2005; Hong et al., 2005), and recent evidence also supports a role for Keap1 in regulation of Nrf2 degradation (Nguyen et al., 2005). A working model based on current knowledge of cellular signaling as it relates to the ARE is presented in Figure 6.4.

To our knowledge, no piscine genes have yet been shown to have functional AREs in their regulatory regions. ARE-like sequences have been identified upstream of the GSTA gene in plaice (*Pleuronectes*

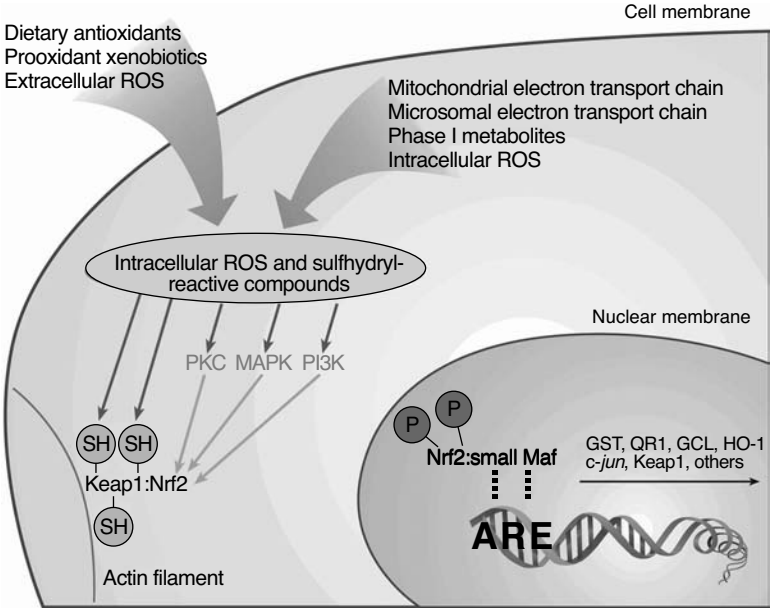

FIGURE 6.4 A working model of ARE-related signaling and gene regulation in mammals, based on references cited in the text. Extracellular and intracellular ROS from any of a number of sources either act directly on the actin-bound Keap1 protein, oxidizing sulfhydryl groups and causing release of Nrf2, or act indirectly via kinase signaling cascades to phosphorylate Nrf2, leading to dissociation of Nrf2 from Keap1 and protection of Nrf2 from proteasomic degradation. Free Nrf2 translocates to the nucleus, heterodimerizes with another protein such as c-Jun or a small Maf protein, and binds to the ARE. This binding facilitates transcription of ARE-driven genes, such as those listed (see also Table 6.1). Note that upregulation of c-*jun* and Keap1, if they occur, may result in positive or negative (respectively) feedback regulation of ARE-mediated gene induction.

platessa) (Leaver and George, 1996; Leaver et al., 1997), and the expression of that gene was inducible by treatment with *trans*-stilbene oxide, β-naphthoflavone, and perfluorooctanoic acid (a peroxisome-proliferating agent) (Leaver et al., 1993, 1997). Three additional lines of evidence support the possibility that some piscine genes are regulated by ARE sequences. First, some of the genes known or hypothesized to be induced by electrophiles in mammals via the ARE have been shown to be inducible by electrophiles in fish species at the levels of mRNA, protein, and catalytic activity. Some examples are presented in Table 6.2. One complication with interpreting these data is the fact that the isoforms of these genes present in fish have not been fully described, making it difficult to know whether the genes seen to be inducible in fish correspond to those known to be ARE regulated in mammals, particularly in the case of catalytic assays. A second line of evidence supporting the likelihood of ARE regulation of endogenous gene expression in fish comes from studies demonstrating the functionality of reporter genes driven by mammalian ARE promoter regions (murine GSTA1, murine QR1, and possibly human QR1) in zebrafish and topminnow cells exposed to a variety of prooxidant chemicals (Carvan et al., 2000, 2001; Rau et al., 2004). A final, and particularly convincing, line of evidence is the demonstration that Nrf2 and Keap1 are present in zebrafish and regulate the expression of zebrafish genes known to be ARE regulated in mammals (GSTP, QR1, and GCLH) (Kobayashi et al., 2002); therefore, the transcription factors necessary to drive ARE-mediated gene transcription in mammals are present and functional in fish, are able to recognize a mammalian ARE sequence, and regulate the expression of endogenous zebrafish genes that are electrophile inducible. These results, although not quite definitive, very strongly suggest that ARE-regulated genes exist in fish as well as mammals and that there is considerable evolutionary conservation of function of ARE-mediated gene regulation between mammals and fish.

TABLE 6.2

Representative List of Gene Products Induced after Exposure to Prooxidants or ROS-Generating Chemicals in Fish Species

Induction Observed at the Level of:	Gene	Refs.
mRNA	GSTP	Kobayashi et al. (2002)
	QR1	Kobayashi et al. (2002)
	GCLC	Kobayashi et al. (2002)
	MT	Kille et al. (1992); Lange et al. (2002); Schlenk et al. (2000)
Protein	GST	Armknecht et al. (1998); Henson et al. (2001); Van Veld et al. (1991)
	Heme oxygenase	Schlenk et al. (1996)
	MnSOD	Meyer et al. (2003)
	Metallothionein	Kille et al. (1992); Pedrajas et al. (1995); Van den Hurk et al. (2000)
Activity	GST	Ahmad et al. (2000); Armknecht et al. (1998); Henson et al. (2001); Stephensen et al. (2002)
	UDPGT	Förlin et al. (1996); Gadagbui et al. (1996); Zhang et al. (1990)
	GCL	Gallagher et al. (1992a); Stephensen et al. (2002)
	QR1	Förlin et al. (1996); Lemaire et al. (1996); Winzer et al. (2002b)
	GR	Åkerman et al. (2003); Förlin et al. (1996); Meyer et al. (2003); Stephensen et al. (2002)
	GPx	Meyer et al. (2003); Radi and Matkovics (1988)
	Heme oxygenase	Ariyoshi et al. (1990)

Note: These genes are candidates for ARE regulation; no gene has yet been conclusively demonstrated to be ARE regulated in any fish species.

ROS-Mediated Modulation of Gene Expression: Summary

The alterations observed in eukaryotic gene regulation in response to ROS are highly complex. These alterations in gene expression may result from interaction with pathways that normally employ ROS as signaling molecules; antioxidant responses, including those aimed at preventing the generation of ROS, increasing the scavenging of ROS, and repair of oxidative damage; and alterations resulting from oxidation of transcription factors or other important cellular macromolecules. The expression of a wide variety of genes is altered by exposure to ROS, including many genes coding for proteins that are not typically thought of as antioxidants, but rarely involves very large changes in expression. These characteristics may relate to the fact that ROS play a normal physiological role in eukaryotes, suggesting that repressing ROS to too low of a concentration might be problematic and to the fact that the transcription factors that mediate many of the apparently adaptive antioxidant responses are involved in other pathways as well, suggesting that large alterations in their activity might be disruptive to other important biological responses.

Deleterious Cellular Effects of Reactive Oxygen Species

As discussed previously, the generation of ROS is a normal consequence of aerobic life and even in some cases a beneficial one. Aerobic organisms have evolved a complex array of antioxidant defenses that effectively deal with typical fluxes of ROS encountered by aerobic cells. As these fluxes increase, due to both natural phenomena (such as increased aerobic metabolism during physical exertion, sharp rises in dissolved oxygen in aquatic environments, or pronounced inflammatory responses) as well as unnatural variables (such as anthropogenic chemicals), cellular antioxidant capacity can be overtaxed. As described above, through mechanisms including ROS-mediated changes in gene expression, cells can adapt to some extent by enhancing their antioxidant capacity. Upregulation of antioxidant defenses comprises an early response to elevated ROS fluxes; however, such fluxes can exceed even adapted

antioxidant capacity, at which point detrimental cellular impacts may ensue. This corresponds to the classic definition first introduced by Sies and Cadenas (1985) of oxidative stress as "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage."

A major difficulty in the study of oxidative stress is the variety of ways at which it can manifest; that is, it is far more difficult to determine if a particular chemical is acting, as a principal mechanism of toxic action, as a prooxidant causing oxidative stress vs. determining, for example, if it is an acetylcholinesterase inhibitor, an aryl hydrocarbon receptor agonist, or an estrogen mimic. A lack of effect as measured by any particular assay (or two or three) cannot be used to rule out the occurrence of oxidative stress. A similar caution applies to the measure of antioxidants as markers for exposure or adaptation to prooxidants, with the expectation that exposures below the threshold for toxicity will result in upregulations of antioxidants. Again, the complexity of mechanisms by which prooxidants may or may not augment various antioxidant system components presents major challenges to this approach. These are issues that have plagued the development of biomarkers for oxidative stress in fish, as described later.

Although oxidative stress is a complex and often difficult subject of inquiry, it remains an important underlying mechanism of cellular toxicity. Given the high reactivity of many ROS, such as ·OH, it is not surprising that they are often indiscriminant in terms of cellular targets. Principal cellular constituents, including lipids, proteins, and DNA, are subject to attack, as are cellular functions such as redox status and energetics and cellular signaling. Tools have been developed to study all of these phenomena. The following is not an exhaustive list of cellular effects but does address major ones that have received considerable research attention in a variety of organisms, including fishes. (Note that oxidative DNA damage is discussed in detail in Chapter 12 and hence is excluded here.)

Lipid Peroxidation

Perhaps the most studied targets of ROS are polyunsaturated fatty acids (PUFAs), which are fatty acids containing two or more carbon–carbon double bonds. Of particular interest are those associated with the membranes of cells, including organelles such as mitochondria, lysosomes, and endoplasmic reticula. These membranes are complex and diverse structures, typically comprised of various phospholipids (such as phosphatidylcholine, or lecithin), cholesterol, lipoproteins, and proteins that serve various functions, particularly with respect to signal transduction and transport of materials across membranes.

In PUFAs, the hydrogen atoms on saturated carbons (allylic hydrogens) adjacent to carbons participating in double bonds are most prone to hydrogen abstraction by oxygen radicals such as ·OH, RO·, and ROO·. These allylic hydrogens form less stable bonds to carbon due to the adjacent carbon–carbon double bonds; an example within a hydrocarbon chain is denoted here in bold:

$$-CH=CH-C\mathbf{H}_2-CH=CH-$$

Abstraction of an allylic hydrogen by an oxygen radical comprises the *initiation* step of lipid peroxidation (Porter et al., 1995; Wagner et al., 1994), which is included in the summary schematic of lipid peroxidation (Figure 6.5); susceptibility of different PUFAs to lipid peroxidation increases with increasing number of unsaturated carbon double bonds. Initiation results in a lipid radical (R·), which then undergoes rearrangement to a conjugated diene radical; under typical aerobic conditions, this lipid radical will readily react with O_2, yielding a lipid peroxyl radical (ROO·). The peroxyl radical can react with another PUFA, thereby abstracting hydrogen, becoming a lipid peroxide (LOOH), and generating another R·. This second R· can also react with O_2 to yield ROO·, and this process can be repeated many times, constituting a free-radical chain reaction termed *propagation* of lipid peroxidation; thus, initiation by one molecule of an oxygen radical can potentially result in the peroxidation of many PUFA molecules. Propagation is an important feature of many free-radical reactions whereby one radical can stimulate a cascade of potentially deleterious reactions in biological systems; this phenomenon is addressed again later.

A number of other important reactions are associated with the process of lipid peroxidation; for example, the peroxyl radical can react with other membrane lipids (e.g., cholesterol) or proteins, in addition to PUFA, thus altering these molecules while forming ROOH. Transition metals such as iron and copper, in addition to enhancing production of the powerful initiator ·OH through Fenton chemistry,

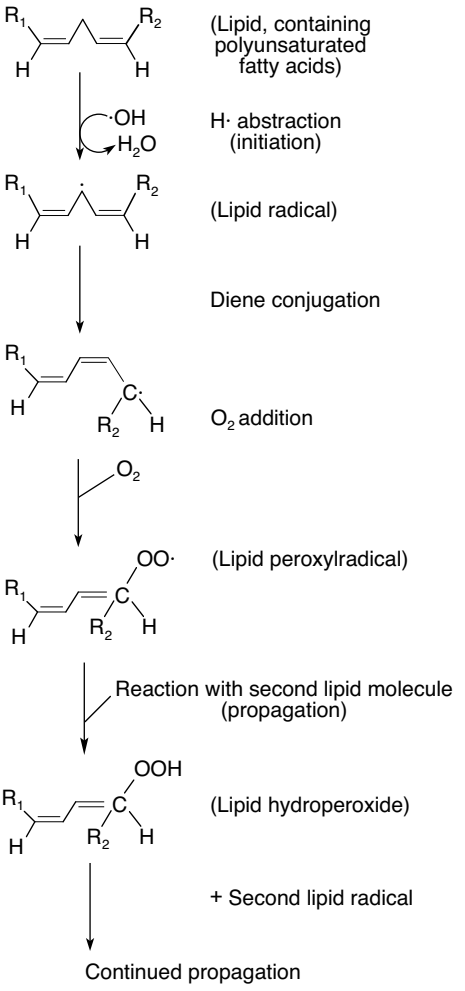

FIGURE 6.5 The biochemical process of hydroxyl radical (·OH)-initiated lipid peroxidation.

can also react directly with ROOH to produce RO· and ROO·, which can initiate new radical chain reactions. In fact, it is thought that transition metals are required for lipid peroxidation to proceed at a significant rate (Sevanian and Ursini, 2000). Lipid peroxidation can be terminated by the reaction of two lipid radicals to produce nonradical products. Additionally, lipid peroxidation can be slowed by the action of α-tocopherol, as described earlier. While stopping a particular radical chain reaction, donation of hydrogen to ROO· by α-tocopherol (yielding the relatively unreactive α-tocopherol radical) results in ROOH, which is still subject to metal-catalyzed radical generation. This is repaired by the action of glutathione peroxidases, discussed earlier, that reduces ROOH to the corresponding alcohol (ROH), effectively preventing further lipid peroxidation. Major consequences of membrane lipid peroxidation include decreased membrane fluidity, increased permeability resulting in inappropriate leakiness to some molecules, and inhibition of membrane-bound enzymes (Richter, 1987).

Protein Oxidations

The effects of ROS on proteins have received less attention than lipid peroxidation, yet it is clear that many proteins are susceptible to attack by ROS, which can have deleterious consequences. Important consequences of protein oxidations by ROS include enzyme inactivation, alteration of receptors and other proteins involved in signal transduction, and perturbed ion homeostasis (via effects on ATPases,

for example) (Halliwell and Gutteridge, 1999). Direct effects of ROS on specific amino acids in proteins include oxidations of the reduced sulfur moiety of cysteine and methionine to produce disulfides and sulfoxides/sulfones, respectively (Dean et al., 1997); the production of carbonyl groups (aldehydes and ketones) on side chains of some amino acids, particularly of proline, arginine, lysine, and threonine (Dalle-Donne et al., 2003); and oxidations of the aromatic side chains of phenylalanine, tryptophan, tyrosine, and histidine (Stadtman and Levine, 2003). Among these effects, the production of carbonyls is considered the most common effect, and increases in their production have been associated with a number of human diseases, including Alzheimer's disease, cystic fibrosis, diabetes, and amyotrophic lateral sclerosis; consequently, many assays have been developed to detect protein carbonyls (Dalle-Donne et al., 2003).

Redox Status and Energetics

The redox status of a cell (or cellular compartment, tissue, etc.) refers to the collective ratios of interconvertible oxidized and reduced forms of redox couples. (For a lucid description and a formal approach for quantifying "redox environments" in the context of cellular oxidative stress, the reader is referred to Schafer and Buettner, 2001.) Key redox couples underlying redox status include $NADH/NAD^+$, $NADPH/NADP^+$, and GSH/GSSG. Reduced forms of these couples are critical for a number of fundamental cellular functions including energy production (e.g., mitochondrial ATP production linked with electron release by NADH, resulting in oxidation to NAD^+), biosynthesis (many pathways employ NADPH), and protection from ROS (wherein GSH plays a central role as described previously); therefore, cells and tissues maintain overall reducing environments that favor the reduced forms of most redox couples, including those just mentioned.

Reactive oxygen species can drive redox status to a more oxidized state through a variety of direct and indirect mechanisms. GSH can be oxidized directly by ROS, as well as through GPX-catalyzed reductions of H_2O_2 and lipid peroxides (Equations 6.15 and 6.16). The reduction of GSSG resulting from GSH oxidations back to GSH is catalyzed by glutathione reductase, which requires the oxidation of NADPH. NADH and NADPH are also oxidized during the activity of quinone reductase (Equation 6.19). Both reduced pyridine nucleotides can be oxidized during the generation of ROS by redox-cycling chemicals, a phenomenon described later. There is also evidence that NADH and NADPH can act directly as ROS scavengers, resulting in their oxidation (Kirsch and De Groot, 2001). This activity is suggested to be important in mitochondria, where their concentrations are similar to that of GSH (~5 m*M*). In all of these cases, however, oxidations of reduced intermediates impose an energetic cost.

Deleterious Organismal Effects of Reactive Oxygen Species

The preceding discussions have focused on molecular and cellular aspects of ROS and oxidative stress, and the complexity and varied nuances of these phenomena should be apparent; therefore, it is not surprising that a diverse array of diseases and pathologies have been associated with oxidative stress. It is beyond the scope of this chapter to discuss these in detail; however, a number of diseases (most described in humans), pathologies, and other effects are noted here to provide a sense of the breadth of health outcomes that are at least in part associated with oxidative stress.

Oxidative stress, as measured by cellular effects described above (e.g., oxidations of lipids, protein, DNA, perturbed redox status, and altered antioxidant capacity), has been observed in association with many disease states and tissue injuries, but it is important to bear in mind that such associations do not confer causality. Evidence of oxidative stress may be encountered in any tissue undergoing trauma due to infection, injury, or cell death (apoptosis or necrosis)—such tissues are inherently more prone to oxidative damage due, for example, to compromised energetics. Determining that oxidative stress is the cause of a disease or tissue damage rather than the result requires careful analysis. Moreover, many diseases have complex etiologies, and attempts to pinpoint a single cause, such as ROS, are often erroneous.

Diseases Associated with Oxidative Stress

Compelling evidence exists that oxidative stress is an important contributing factor for numerous diseases and pathologies. Atherosclerosis, for example, is a disease of the arteries characterized by the thickening of the vessel walls; it is a major contributor to heart attacks and stroke, which are the leading causes of death in the United States and Europe. The causes and progression of the disease are complex and not fully understood, but ample evidence implicates ROS and RNS as important components (Halliwell and Gutteridge, 1999; Harrison et al., 2003). Also, chronic inflammatory diseases such as arthritis are generally believed to be in part caused by ROS produced by activated phagocytes at inflamed sites, in part an autoimmune response (Closa and Folch-Puy, 2004). The brain is considered particularly prone to oxidative stress due to its high rate of oxidative metabolism, presence of autooxidizable neurotransmitters (dopamine and norepinephrine), high levels of highly unsaturated fatty acids, relatively modest antioxidant defenses, and presence of cytochrome P450s (Halliwell and Gutteridge, 1999). Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis are believed to be in part due to ROS, although precise mechanisms remain unresolved (Anderson, 2004). Cancer encompasses a diverse set of diseases with a complex etiology and has been studied extensively in fishes as well as humans. Considerable evidence supports a role for ROS and other free radicals in tumor initiation, promotion, and progression (Klaunig et al., 1998); relevant studies in fish models are described in Chapter 12.

In addition to these and other diseases, an important pathology closely associated with oxidative stress is referred to as *ischemia-reperfusion tissue injury*. Ischemia refers to a loss of blood flow to a tissue, a key consequence of which is hypoxia. Hypoxia can result from atherosclerosis, physical tissue damage, surgical procedures, and depletion of environmental O_2. The effects of hypoxia are variable, depending on severity, time course, and tissue involved and can range from mild and reversible to irreversible tissue damage to death. A somewhat paradoxical aspect of ischemia is that sometimes tissue injury coincides with reperfusion (i.e., restoration of blood flow and normoxia) (Halliwell and Gutteridge, 1999). This injury is associated with oxidative stress due to cellular changes such as ATP depletion and enhanced ROS generation via the xanthine oxidase system (McCord, 1987) and altered mitochondrial enzyme activities (Powell and Jackson, 2003). Evidence also suggests that an environmental analogy to this phenomenon may occur in some aquatic systems such as shallow lakes and estuaries that display marked diurnal and seasonal fluctuations in dissolved oxygen levels (Hermes-Lima and Zenteno-Savin, 2002; Hochachka et al., 1996).

Aging and Oxidative Stress

The processes that govern the aging process and determine the limits of lifespan of organisms have been the subject of debate and research for many years. Considerable evidence supports an important role for oxidative stress in these phenomena, although other factors are likely involved (Beckman and Ames, 1998; Finkel and Holbrook, 2000). Supporting evidence for the free-radical theory of aging includes the increased accumulation of oxidized proteins, lipids, and DNA with age; increased lifespan in organisms engineered to overexpress antioxidant enzymes (e.g., CuZnSOD and GR) in some studies; and increased lifespan associated with caloric restriction (which reduces mitochondrial production of ROS, a key component of this theory). As described earlier, ROS modulates several cellular signaling pathways, including stress-responsive pathways considered adaptive to oxidative stress. The responsiveness of some pathways, such as heat shock proteins, appears to decline with age. Fish display a range of patterns with respect to aging, including rapid senescence in some salmonids, and may provide useful models for ageing research.

Chemicals and Oxidative Stress

Numerous chemicals including natural products, drugs and environmental contaminants have been shown to impact organismal health via, at least in part, oxidative stress. Some better understood examples, largely from mammalian studies, are noted in the following text.

Natural Products

The Gram-negative bacterium *Pseudomonas aeruginosa* can cause pneumonia via secretion of two compounds: procyanin and pyochelin (5-methyl-1-hydroxyphenazine). Procyanin generates $O_2^{\bullet-}$ via redox cycling (described in the following section), and pyochelin is a siderophore that binds iron and promotes Fenton-chemistry-like generation of ·OH (Britigan et al., 1992). Juglone (5-hydroxy-1,4-naphthoflavone; see Figure 6.2) and plumbagin (5-hydroxy-3-methyl-1,4-naphthoflavone) are redox-cycling quinones produced by walnut trees (*Juglans* spp.). They suppress germination and growth of other plant species (hence, the term *allelotoxins*), and their use in cosmetics has raised concerns for human health (Inbaraj and Chignell, 2004; Seguraaguilar et al., 1992). In part, the hepatotoxicity of ethanol, a byproduct of glucose metabolism by yeast, is believed to be associated with ethanol-mediated induction of cytochrome P4502E1 (CYP2E1) and a resulting shift in ethanol metabolism to include a greater contribution by CYP2E1 vs. the dominant pathway (alcohol and acetaldehyde dehydrogenases). CYP2E1 is a relatively leaky P450, and substantial $O_2^{\bullet-}$ and H_2O_2 are generated as a result of its activity, which can thereby contribute to oxidative stress in the liver during chronic ethanol exposure (Caro and Cederbaum, 2004).

Drugs

The deleterious side effects of some drugs are associated with oxidative stress. The liver and kidney damage produced by high dosages of the common pain reliever acetaminophen occurs via GSH depletion by the drug and a CYP2E1 metabolite (*N*-acetyl-*p*-benzoquinone) and accompanying effects on calcium metabolism (Moore et al., 1985). Acetaminophen has been used frequently in suicide attempts, underscoring its toxic nature at high doses. The common urinary tract antibacterial nitrofurantoin is a nitroaromatic, and both its bactericidal activity and major deleterious side effect (lung damage) are thought to be due to ROS generation via redox cycling through a nitro radical intermediate (Suntres and Shek, 1992). A number of antibiotics, such as penicillins, natamycin, tetracyclines, streptonigrin, and gentamicin, have been shown capable of generating ROS that are believed to underlie the tissue damage that sometimes accompanies their use (Halliwell and Gutteridge, 1999). The addictive recreational drug cocaine is a hepatotoxicant and neurotoxicant that is thought to act in part via ROS-generating reactive metabolites (Boelsterli and Goldin, 1991), as well as through vasoconstriction that can also exert oxidative stress, as indicated by elevated oxidations of glutathione and α-tocopherol (Lipton et al., 2003).

Environmental Pollutants

Numerous environmental pollutants have been demonstrated to impact organismal health via oxidative stress; several illustrative examples are described here. Carbon tetrachloride (CCl_4) is a common solvent and intermediate in the production of other chemicals and was the first environmental toxin to be shown to exert toxicity through a free radical mechanism. Many of its uses, including as a solvent and cleaning agent, have declined since the 1970s due largely to its great potency as a hepatic and renal toxicant, as well as its carcinogenicity and contributing to stratospheric ozone depletion (ATSDR, 1994). Its toxicity is based on its conversion by cytochrome P450s, particularly CYP2E1, to a trichloromethyl radical ($CCl_3^{\bullet}$) (Recknagel et al., 1989; Zangar et al., 2000). This radical can bind to proteins and lipids directly and also react with O_2 to produce the thrichloromethylperoxyl radical ($Cl_3COO^{\bullet}$). Both processes lead to extensive membrane damage, including lipid peroxidation, and can lead to elevated intracellular Ca^{2+}, an important component of CCl_4 cytotoxicity (Stoyanovsky and Cederbaum, 1996).

Benzene is a ubiquitous contaminant used as a precursor for various products; benzene is particularly toxic to mammalian bone marrow, and epidemiological studies indicate that elevated exposures cause leukemias in humans, as well as other cancers (Snyder, 2002). The metabolism of benzene to toxic intermediates is also initiated by cytochrome P450s, particularly CYP2E1, which yield various phenols. These phenols are distributed to various tissues, where additional metabolism can yield highly redox-active quinones via the action of various peroxidases, such as myeloperoxidase, which is highly enriched in bone marrow. Quinones comprise a large set of reactive intermediates of numerous compounds, including PAHs, estrogens, and catecholamines, that have been shown collectively to produce acute

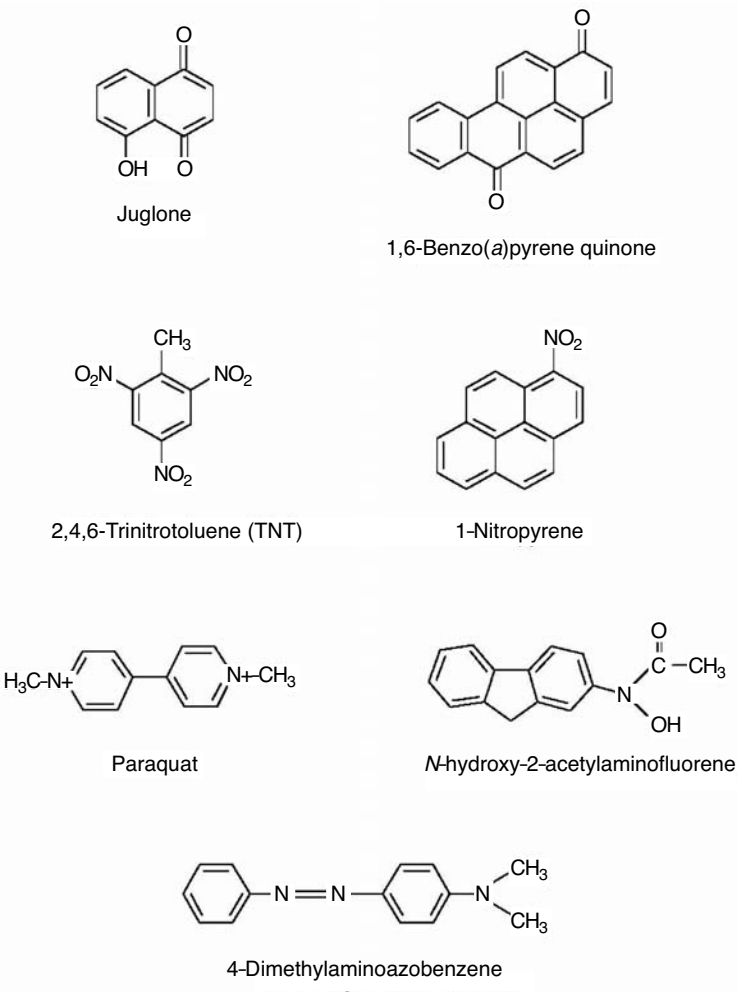

FIGURE 6.6 Representative redox-cycling chemicals including a natural product (juglone), a metabolite of the procarcinogen benzo(*a*)pyrene (1,6-benzo(*a*)pyrene quinone), the explosive 2,4,6-trinitrotoluene (TNT), a common combustion byproduct (1-nitropyrene), an herbicide (paraquat), a carcinogenic aromatic hydroxylamine (*N*-hydroxy-2-acetylaminofluorene), and an azo dye (4-dimethylaminobenzene).

cytotoxicity, immunotoxicity, and carcinogenicity (Bolton et al., 2000). Quinone toxicity can result from ROS generation via redox cycling as well as from direct alkylations of protein and DNA.

Paraquat (Figure 6.6) and diquat are broad-spectrum, bipyridyl-based herbicides that have been used extensively in no-till farming and marijuana control (paraquat) and for the control of nuisance aquatic plants (diquat). The toxicity of these compounds to plants is based on their efficiency as redox cyclers in chloroplasts that copiously transfer electrons and generate O_2 during photosynthesis (Bowler et al., 1994). Their toxicity to animals also involves their ability to redox cycle and produce oxidative stress. Paraquat is unusual in that, regardless of route of exposure in mammals, it is selectively toxic to lung tissue. This phenomenon appears to be due to its propensity to be actively transported by systems involved in the transport of polyamines such as putrescine (Smith and Wyatt, 1981). Polyamines are endogenous compounds that, like paraquat, contain positively charged quaternary nitrogen atoms. They are involved in cellular growth and differentiation and are actively taken up by lung cells; thus, the selectivity of paraquat to lung tissue appears to be due to its active transport by these cells, which have high O_2 concentrations that facilitate redox cycling.

Mechanisms of Chemical-Mediated Oxidative Stress

As mentioned earlier, oxidative stress has been defined as "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage" (Sies, 1985). Following this definition, two broad sets of mechanisms by which pollutants can produce oxidative stress can be delineated: mechanisms that enhance ROS production and mechanisms that reduce antioxidant capacity. Illustrative examples of both are described below.

Enhanced ROS Production

Mechanisms by which chemicals can enhance ROS (and in some cases NOS) production include redox cycling, interactions with electron transport chains (notably in mitochondria and microsomes, as well as chloroplasts in plants), and photosensitization. Additionally, some chemicals are metabolized to carbon-based free radicals that can donate unpaired electrons to O_2 thereby generating $O_2^{\bullet -}$; CCl_4 (described above) is a notable example. Moreover, some air pollutants occur as radicals themselves, such as nitrogen dioxide, $NO_2^{\bullet}$.

Redox Cycling

Redox cycling is perhaps the most common mechanism by which a diverse array of chemicals including many environmental pollutants can generate intracellular ROS. Redox cycling chemicals include diphenols and quinones, nitroaromatics and azo compounds, aromatic hydroxylamines, bipyridyliums, and certain metal chelates, particularly of copper and iron (Di Giulio et al., 1989, Halliwell and Gutteridge, 1999). These include large classes of compounds of broad industrial use, many pesticides, ubiquitous elements, and metabolic products of numerous pollutants. Examples of these redox active chemicals are provided in Figure 6.6.

In the redox cycle, the parent compound accepts an electron from a reduced cofactor, such as NADH or NADPH; this reaction is typically catalyzed by a reductase such as xanthine oxidase or cytochrome P450 reductase (Kappus, 1986). Cytochrome P450 reductase normally functions to transfer electrons from NADPH to cytochrome P450 via FAD and flavin-mononucleotide (FMN) contained in this reductase (Chapter 4). However it is also a key catalyst in xenobiotic redox cycling in which the electron donated by NADPH yields a radical of the xenobiotic. In the presence of O_2, the unpaired electron of the radical metabolite is donated to O_2, yielding $O_2^{\bullet -}$ and regenerating the parent compound; importantly, the parent compound can repeat this cycle until it is cleared or metabolized to an inactive product. In the course of each redox cycle, two potentially deleterious events occur—a high-energy reducing equivalent is expended (the oxidation of NADPH to NAD^+, for example), and an oxygen radical is produced. Moreover, the proliferative nature of these potentially harmful outcomes associated with redox cycling (i.e., one molecule of xenobiotic causing the oxidation of many molecules of NADPH and the production of many molecules of $O_2^{\bullet -}$) is observed in other aspects of free radical biology, such as lipid peroxidation described earlier. Redox cycling of some chemicals also occurs in the mitochondria (Doroshow and Davies, 1986; Cadenas and Davies, 2000). A generalized redox cycle that includes associations with cellular toxicities and antioxidant defenses is shown in Figure 6.7. It should be noted, however, that in some cases redox cycling chemicals are toxic by mechanisms other than ROS production (Imlay and Fridovich, 1992); thus, the *in vitro* capability of a chemical to cause oxidative stress is not a definitive demonstration of an *in vivo* mechanism of toxicity.

Uncoupling or Inhibition of Electron Transport

Another important mechanism by which chemicals can enhance ROS production is through interactions with electron transport chains, particularly those in mitochondria and endoplasmic reticula (microsomes) in animals. As discussed earlier, these systems are important sources of ROS during normal aerobic respiration due a degree of inherent uncoupling between NAD(P)H oxidation and substrate reduction.

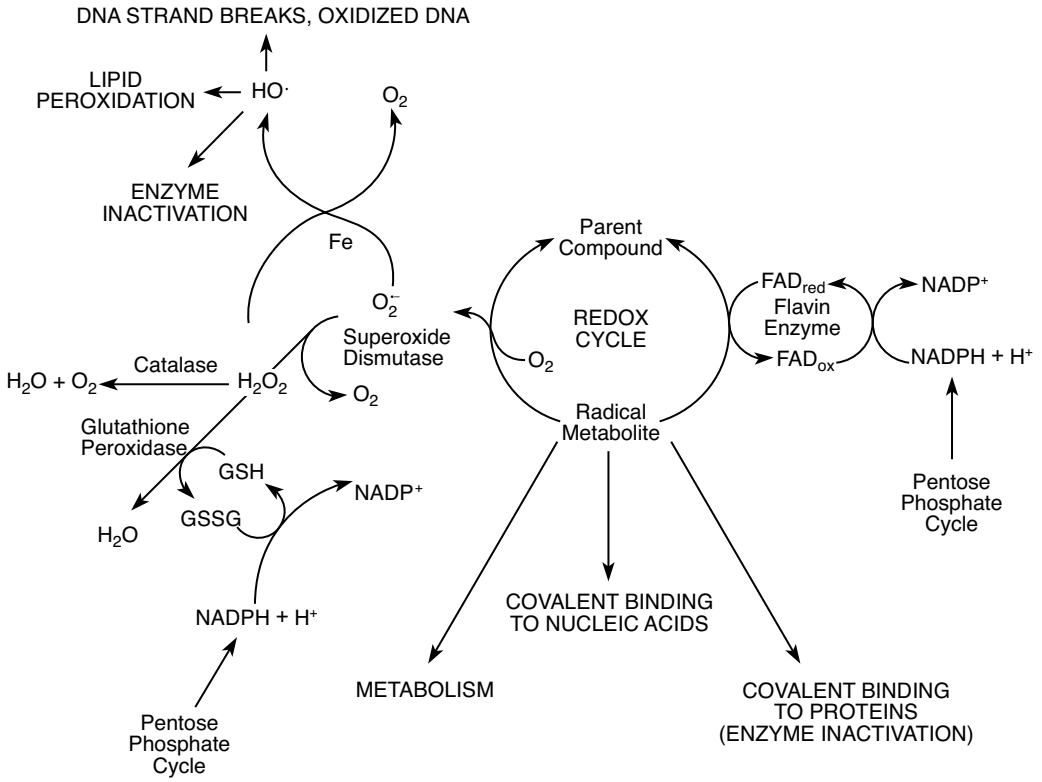

FIGURE 6.7 An overview of ROS generation by redox cycling, key enzymatic antioxidant defenses, and cellular targets of ROS. (Adapted from Kappus, H., in *Oxidative Stress*, Sies, H., Ed., Academic Press, London, 1985, pp. 273–310.)

Chemicals can enhance ROS production by these chains in several ways. Planar halogenated aromatic hydrocarbons (PHAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and certain polychlorinated biphenyls (PCBs) that bind to the AhR and upregulate expression of genes for biotransformation enzymes including CYP1A (see Chapter 4) have been observed to enhance ROS generation in mitochondria, microsomes, and whole cells (Nebert et al., 2000; Park et al., 1996). In the case of TCDD-enhanced H_2O_2 production in mice mitochondria, this effect was found to be dependent on TCDD binding to the AhR but independent of CYP1A activity (Senft et al., 2002). Studies with 3,3′,4,4′-PCB and 3,3′,4,4′,5-PCB (PCB 77 and PCB 126, respectively, both established AhR ligands) in the marine fish scup (*Stenotomus chrysops*) demonstrated the ability of these AhR agonists to potently induce CYP1A and at higher doses to uncouple microsomal electron transfer, resulting in increased ROS production and inactivation of CYP1A (Schlezinger and Stegeman, 2001; Schlezinger et al., 1999). In this case, the production of ROS was thought to be associated with interactions of the active site of the enzyme with these chlorinated chemicals that are recalcitrant to metabolism and thereby uncouple normal electron flow to the substrate (i.e., PCB), resulting in ROS.

Inhibition of electron transport in mitochondria can also lead to production of ROS; blockage of the flow of electrons leaves mitochondrial proteins in a highly reduced state, which can lead to the reduction of oxygen and ROS production. Chemicals known to produce ROS by this mechanism include rotenone, an insecticide (Li et al., 2003); antimycin A, an antibiotic frequently used to study inhibition of mitochondrial electron transport (Chen et al., 2003); 1-methyl-4-phenylpyridinium (MPTP), a compound used to study Parkinson's disease (Smith and Bennett, 1997); certain oxidatively modified PAHs (Tripuranthakam et al., 1999); and cadmium (Wang et al., 2004). In some cases, however, xenobiotic-mediated uncoupling of electron transport in mitochondria can result in reduced production of ROS (Kowaltowski and Vercesi, 1999; Turrens, 1997).

Photosensitization

Ultraviolet (UV) radiation is typically divided into three general categories: UVC (200 to 280 nm), UVB (280 to 320 nm), and UVA (320 to 400 nm). UVC is not environmentally relevant, as it is effectively blocked by the Earth's atmosphere; however, UVB and UVA radiation to differing degrees do have the ability to penetrate the Earth's atmosphere and water columns to depths dependent on the wavelength of the radiation and the clarity of the water. Both are capable of generating ROS and free radicals either directly or via excitation of photosensitizing chemicals, including both endogenously produced compounds (Young, 1997) and many common pollutants of aquatic systems (Larson and Weber, 1994). Many polycyclic aromatic hydrocarbons (PAHs), for example, are orders of magnitude more acutely toxic to aquatic organisms, including fish, in the presence of ultraviolet radiation than in its absence (Ankley et al., 1997; Arfsten et al., 1996; Bowling et al., 1983; El-Alawi et al., 2002). Energy absorbed by the chemical bonds present in these compounds excites electrons to higher energy orbitals; this energy can lead to changes in the chemical structure of the PAH and in some cases reaction with other molecules, such as oxygen, in a process referred to as *photomodification* (Huang et al., 1997; McConkey et al., 1997). The extra energy can also cause the loss of an electron from the PAH, or the extra energy can be given off by the compound in a variety of ways (Larson and Weber, 1994; Schwarzenbach et al., 1993). As an example, photosensitization occurs when the energy gained by the PAH that absorbed the photon (the photosensitizer) is then passed on to another molecule that would not have been chemically able to absorb that energy directly from the photon, such as O_2 (El-Alawi et al., 2002; Schwarzenbach et al., 1993). Both photosensitization and photomodification of PAHs can lead to the production of ROS; for example, when an electron is lost, it is often absorbed by oxygen, leading to the production both of superoxide anion and a radical compound. Similarly, when energy is lost from the excited compound, it can be transferred to oxygen, producing the reactive singlet oxygen, which is highly reactive and toxic (Briviba et al., 1997). Of course, high-energy radiation such as UV radiation can cause damage in the absence of photosensitizers; however, although UV radiation has been shown to cause oxidative stress, DNA damage, and other biological effects in fish (Charron et al., 2000; Fabacher et al., 1994; Lesser et al., 2001), the environmental relevance of this source of oxidative stress to fish is unclear (Williamson, 1995).

Diminished Antioxidant Defense

Although less studied than enhanced ROS production, interference with antioxidant defense system components represents another mechanism by which chemicals can exert oxidative stress in animals. Inhibition of antioxidant enzymes comprises an important component of this phenomenon; for example, quinones have been shown to inhibit SOD activities (Smith and Evans, 1984). This effect may be mediated by H_2O_2 generated via redox cycling, as prolonged H_2O_2 exposure can inhibit CuZnSOD and FeSOD (Halliwell and Gutteridge, 1999). Relatedly, extracts of diesel exhaust particles were observed to effectively inhibit CuZnSOD *in vitro*, and this effect was surmised to be associated with quinone components of the extracts (Kumagai et al., 1995). Dowla et al. (1996) examined the effects of several chemicals used in agriculture (acephate, cadmium, methamidophos, maleic hydrazide, and nicotine) on activities of human blood SOD, cholinesterase, and δ-aminolevulinic acid dehydrase (ALAD) and found SOD to be the most sensitive to inhibition by all chemicals tested. Plant polyphenols such as chalcones and tannic acids were shown to be potent GR inhibitors *in vitro*, with IC_{50} values in the micromolar range (Zhang et al., 1997). The relevance of such *in vitro* studies to *in vivo* exposures remains speculative. Methylmercury, however, was shown to inhibit hepatic GPX activities and reduce GSH levels in rat pups; these effects were associated with increased lipid peroxidation and hepatotoxicity. These results support the purported prooxidant role of methylmercury, a metal complex that cannot directly generate ROS via redox cycling or Fenton chemistry, as is the case for metals such as Fe and Cu. Moreover, the reactive nature of the sulfhydryl group of GSH makes this critical antioxidant prone to depletion by a number of oxidants and electrophiles.

Studies with Fishes

A large number of studies of oxidative stress in fish have been carried out in the last 25 years or so, with different motivations (Di Giulio et al., 1989). These motivations might be summarized as: (1) increasing the understanding of basic biochemical and molecular mechanisms related to oxidative stress in fish, (2) developing biomarkers related to oxidative stress fish, and (3) investigating the relationships between oxidative stress and the health of wild populations of fish. Some of the results of the studies carried out are presented below with respect to these three concerns.

Basic Biochemical and Molecular Mechanisms of Oxidative Stress in Fish

The study of the toxicity of prooxidant xenobiotics in fish serves both to inform risk assessment with regard to fish populations and to inform our understanding of the toxicity of such chemicals in other organisms, including humans (Bailey et al., 1996; Di Giulio et al., 1989; Kelly et al., 1998; Winston, 1991). Mechanistic studies in fish can be further categorized as characterizing the ability of a xenobiotic to generate ROS in fish cells or living fish, as well as characterizing the antioxidant defenses present in different species of fish, either with or without prior xenobiotic exposure. It is often useful to employ comparative studies involving different species (piscine and nonpiscine) to better understand the impact of prooxidant chemicals in the environment.

In Vitro Biochemical Generation of ROS

In vitro studies have clearly demonstrated that ROS are generated in subcellular fractions (microsomal, mitochondrial, and cytosolic) of fish tissues. Generation of ROS in fish, as in mammals, is sometimes involved in normal physiological processes such as immune function (Anderson, 1994); moreover, release of ROS by phagocytic cells can be stimulated after pollutant exposure in fish (Fatima et al., 2000). In addition, laboratory and field exposures to prooxidant chemicals have resulted in increased ROS production in subcellular fractions of various tissues, particularly liver, gill, and kidney (Lemaire et al., 1994; Livingstone, 2001; Livingstone et al., 2000; Peters et al., 1996; Schmieder et al., 2003; Washburn and Di Giulio, 1988). As discussed previously in this chapter, xenobiotics have been shown *in vitro* to enhance ROS production and oxidative stress by diverse mechanisms, including redox cycling, depletion of glutathione or other antioxidants, facilitation of Fenton chemistry, uncoupling of mitochondrial electron transport, and uncoupling of cytochrome P4501A monooxygenation reactions.

Enzymatic Antioxidant Defenses in Fish

Studies aimed at understanding the biochemistry and, to a much lesser degree, the molecular biology of fish antioxidant systems have also been carried out. Many if not all of the basic antioxidant defenses (enzymatic and nonenzymatic) characterized in mammals are also present in fish. Representative studies describing antioxidant enzymatic activities, reactivity of mammalian antibodies to presumably homologous fish antioxidant enzymes, and sequences for antioxidant genes in fish are presented in Table 6.3. In addition, similar evidence supports the existence of many of the phase II enzymes that can be viewed as playing an antioxidant role or that are regulated by an ARE in mammals (discussed earlier in the section on the ARE and in Chapter 4). Certain GST isoforms in mammals, for example, may play an antioxidant role both by conjugating electrophilic compounds in general (Chapter 4) and by metabolizing 4-hydroxy-2-nonenal (HNE) (Eaton and Bammler, 1999). HNE is a lipid peroxidation product that is both highly toxic and involved in ROS-related signaling (Poli and Schaur, 2000); recent evidence indicates that fish species also express a GST isoform capable of metabolizing HNE (Leaver and George, 1998; Pham et al., 2002; 2004). Similarly, there is evidence that in fish, as in mammals, some GST isoforms possess a glutathione peroxidase activity (Martínez-Lara et al., 2002).

Unfortunately, the exact isoforms of antioxidant enzymes present in different fish species have rarely been carefully identified and characterized. In most cases, enzyme activities have been measured with slight adaptations of assays developed with mammalian species and may reflect the activities of multiple

TABLE 6.3

Representative List of Some of the Antioxidant Enzymes Identified and Partially Characterized in Studies with Fish

Characterization of Antioxidant Gene Product	Gene	Refs.
Gene sequence (full or partial)	GPX1 and GPX4	Kryukov and Gladyshev (2000)
	Catalase	Gerhard et al. (2000)
	MT	Kille et al. (1992); Schlenk et al. (1996); Scudiero et al. (2001); Yan and Chan (2002)
	QR1	Kobayashi et al. (2002)
	GCLC	Kobayashi et al. (2002)
	GR	GenBank
	CuZnSOD	GenBank
	Thioredoxin peroxidase	GenBank
	Heme oxygenase	GenBank
Antibody reactivity or protein characterization	MnSOD	Orbea et al. (2000); Meyer et al. (2003)
	GPx	Nagai et al. (2002); Orbea et al. (2000)
	Heme oxygenase	Schlenk et al. (1996)
	Catalase	Braunbeck et al. (1991); Orbea et al. (2000)
	CuZnSOD	Nakano et al. (1995); Capo et al. (1997); Orbea et al. (2000); Meyer et al. (2003)
	MT	Van den Hurk et al. (2000)
Enzyme activity	QR1	Hasspieler and Di Giulio (1992)
	GR	Gallagher and Di Giulio (1992)
	GCL	Gallagher and Di Giulio (1992)
	GPx	Gallagher and Di Giulio (1992); Nagai et al. (2002)
	Glutathione transpeptidase	Wallace (1989); Gallagher and Di Giulio (1992)
	CuZnSOD	Matkovics et al. (1977); Capo et al. (1997)
	Catalase	Matkovics et al. (1977); Förlin et al. (1995); Dorval and Hontela (2003)

Note: Sequences submitted to GenBank but not yet published in the literature are included only when no sequence has been published but are in some cases also available in other species for genes whose sequences have been published. Genes not traditionally considered antioxidant but that may play a certain antioxidant role (e.g., GSTs or UDPGTs, as discussed in the text) are not included in this table.

enzymes. As a result, the number of enzymes performing specific catalytic activities, their molecular regulation, and their biochemical characteristics are usually unknown. Studies that have been carried out with genes related to xenobiotic metabolism such as the UGTs (Clarke et al., 1992; George and Taylor, 2002), GSTs (Gadagbui and James, 2000; Leaver et al., 1997; Ramage and Nimmo, 1984), and AhR pathways (Hahn, 2002), as well as other gene families such as the Hox genes (McClintock et al., 2001) in fish, suggest that multiple isoenzymes will be discovered in many fish species, perhaps in some cases more than observed in mammals. Some evidence already exists supporting the possibility of multiple antioxidant isoenzymes such as glutathione peroxidases (Kryukov and Gladyshev, 2000) and metallothioneins (Bargelloni et al., 1999) in fish. Unique catalytic properties that may belong to novel enzymes have been identified in fish species (Hasspieler and Di Giulio, 1994). A theoretical reason to expect multiple antioxidant enzymes in fish is that many fish species are tetraploid because of a chromosome duplication event that occurred after the phylogenetic divergence of ray-finned fishes from other vertebrates (Carroll, 1988). Furthermore, in addition to differences between mammals and fishes, it is likely that very large differences will be identified among different fish species, given the long time over which fish species have been evolving.

Nonenzymatic Antioxidant Defenses in Fish

The major nonenzymatic antioxidant defenses observed in mammals have also been studied in fish. By far the most studied from a toxicological perspective is glutathione, which is present in fish tissues at levels comparable to those observed in mammals (e.g., millimolar concentrations in liver tissue) (Nimmo, 1987). As observed in mammals, tissue glutathione levels are often depleted after short-term oxidant exposures but elevated after long-term exposures. Furthermore, glutathione depletion sensitizes fish, like mammals, to the toxicity of prooxidant xenobiotics (Gallagher et al., 1992a). Other important antioxidants, such as vitamin C, vitamin E, ubiquinone, and carotenoids, have been less studied relative to toxicology in fish but likely play an important role (Bell et al., 2000; Olsen et al., 1999; Parihar and Dubey, 1995; Payne et al., 1998; Tocher et al., 2002). In addition, important differences clearly exist in terms of the ability of different fish species to synthesize vitamin C (Moreau-Regis and Dabrowski, 1998). Although the majority of studies of nonenzymatic antioxidants in toxicology have historically focused on glutathione, it is increasingly clear that other compounds are also important. Studies of the total oxyradical scavenging capacity (TOSC) (Regoli and Winston, 1999; Winston et al., 1998) of tissue homogenates from both aquatic and nonaquatic organisms have demonstrated that compounds other than the classical antioxidants such as glutathione, vitamin C, and vitamin E can contribute very significantly to the *in vitro* oxyradical scavenging capacity of those tissue preparations. As in mammals, it is likely that diet plays a very important role in determining the nonenzymatic antioxidant capacity of fish tissues (Mourente et al., 2000; Nakano et al., 1999; Olsen et al., 1999; Pascual et al., 2003).

In Vivo Prooxidant Studies in Fish

As previously stated, the fact that a chemical produces ROS in an *in vitro* situation does not mean that the same chemical will cause oxidative stress *in vivo*. The additional complications introduced by kinetics of uptake, metabolism, and excretion in a living organism can be understood only by testing the effect of a chemical in an *in vivo* system. Table 6.4 is a representative list of stressors shown to exert oxidative stress either in intact fish or, in a few cases, in fish cells. It is important to bear in mind that not all chemicals in the chemical classes listed in column 1 are expected to be prooxidants. Specific chemicals that induced oxidative stress in the studies cited are listed in parentheses as examples. Generally speaking, the chemicals shown to cause oxidative stress in mammals have also done so in fish.

Many chemicals are more or less toxic depending on other factors. An example that has received considerable attention recently is phototoxicity, which is the greatly enhanced toxicity of specific chemicals (especially many PAHs) in the presence of ultraviolet radiation. UV radiation can excite an electron of such chemicals, and the resultant excited-state molecule is more reactive and often ultimately more toxic. Empirical studies with fish have strongly supported the hypothesis that the phototoxicity or photo-enhanced toxicity of PAHs resulting from the process of photosensitization is mediated by oxidative stress (Choi and Oris, 2000; Weinstein et al., 1997). In addition, chemical structure alterations produced by UV irradiation can produce compounds such as quinones and phenols that are capable of redox cycling and interfering with electron flow in electron transport chains of mitochondria (Huang et al., 1997; Tripuranthakam et al., 1999), both processes that can lead to oxidative stress. Thus, phototoxicity represents a distinctive source of oxidative stress that may be of particular importance in aquatic systems with clear water columns. It is important to note, however, that most of the studies carried out thus far have been conducted under laboratory or constrained field conditions, so the ecological relevance of phototoxicity is controversial (McDonald and Chapman, 2002).

Differences between Fish and Mammals

As we have seen, many of the basic mechanisms of ROS production and damage are shared between fish and mammals. Despite the overall similarities in antioxidant defenses, however, important differences exist between fish and mammalian species, some of which are summarized here. ROS production in microsomal fractions was higher in rainbow trout, as well as in two aquatic invertebrate species, with

TABLE 6.4

Representative List of Stressors (Chemical and Nonchemical) Shown to Cause Oxidative Stress,[a] Induce Production of Reactive Oxygen Species,[b] or Induce or Repress Antioxidant Defenses

Stressor (Common Examples)	Refs.
Hydrogen peroxide	Winzer et al. (2000, 2001)
Quinones (menadione, naphthoquinone, benzoquinone)	Akerman et al. (2003); Petrivalsky et al. (1997); Schmieder et al. (2003); Stephensen et al. (2002)
Aromatic nitro compounds (nitrofurantoin)	Winzer et al. (2001)
Metals (cadmium, copper)	Ariyoshi et al. (1990); Carvan et al. (2001); Pedrajas et al. (1995); Radi and Matkovics (1988); Rau et al. (2004)
PAHs (β-naphthoflavone, benzo(*a*)pyrene, 3-methylcholanthrene)	Carvan et al. (2001); Hughes and Gallagher (2004); Stephensen et al. (2002); Winzer et al. (2000, 2001)
Pesticides (paraquat, endosulfan, dieldrin, malathion, chlorothalonil)	Åkerman et al. (2003); Dorval et al. (2003); Dorval and Hontela (2003); Elia et al. (2002); Gallagher et al. (1992); Luo et al. (2005); Pandey et al. (2001); Pedrajas et al. (1995)
Halogenated aromatic hydrocarbons (PCBs, dioxins)	Carvan et al. (2001); Förlin et al. (1996); Schlezinger and Stegeman (2001); Schlezinger et al. (1999)
Ozone	Ritola et al. (2002)
Hypoxia	Cooper et al. (2002)
Hyperoxia	Ritola et al. (2002)
Anoxia-reoxygenation	Lushchak et al. (2001); Ross et al. (2001)
Heat stress	Parihar and Dubey (1995); Parihar et al. (1996)

[a] As indicated by markers such as lipid peroxidation.

[b] As indicated *in vitro* either by dyes whose fluorescent properties are altered by exposure to ROS or by reporter transgenes.

Note: Not all chemicals pertaining to the chemical classes listed are prooxidant; specific examples studied in the references cited are listed in parentheses.

NADH than with NADPH as a source of reducing power, in contrast to the situation seen in rats (Jewell and Winston, 1989; Kennish et al., 1989). Glutathione depletion under conditions of starvation was much slower in mullet (*Mugil cephalus*) (Thomas and Wofford, 1984) and channel catfish (*Ictalurus punctatus*) (Gallagher et al., 1992b) than in rodents. Perhaps because of the observed low (relative to rodents) rate of turnover of glutathione in catfish, administration of buthionine sulfoximine (BSO), an inhibitor of GCL, did not completely deplete hepatic glutathione, although coadministration of BSO and diethyl maleate (DEM), a glutathione depletor, led to nearly complete depletion of glutathione (Gallagher et al., 1992b). There is considerable variability in the reported catalytic activities of many of the antioxidant enzymes, both between fish and mammals and between different fish species (Di Giulio et al., 1989; Forlin et al., 1995; Gadagbui et al., 1996; Hasspieler et al., 1994a,b; Ploch et al., 1999; Rana and Singh, 1996; Winston, 1991). Biochemical differences that are important in terms of sample preparation and treatment also exist; for example, mammalian CuZnSOD is a remarkably thermostable enzyme, but the CuZnSODs from two different fish species are much more labile at high temperatures (60°C and above) (Capo et al., 1997; Nakano et al., 1995). Vitamin E levels appear to be generally higher in fish cell membranes than in mammalian cell membranes, but it is unclear whether this difference is protective in the context of the typically higher levels of PUFA observed in fish cell membranes (Bell et al., 2000; Singh et al., 1992). Some studies have found that fish cells are more resistant than mammalian cells to oxidative stress *in vitro* (Singh et al., 1992; Venditti et al., 1999), while others have found the opposite (Rau et al., 2004).

Biomarkers of Oxidative Stress

How is it possible to know if oxidative stress is a problem in a given field situation? Many studies have attempted to identify and evaluate reliable biomarkers of exposure to pollution (see Chapter 16). Despite a large number of laboratory exposures to pure compounds (Almeida et al., 2004; Bergman et al., 1994; de Pandey et al., 2001; Dorval et al., 2003; Rau et al., 2004) and environmentally relevant contaminant mixtures (Bergman et al., 1994; Celander et al., 1994; Di Giulio et al., 1993; Livingstone et al., 1992; Meyer et al., 2003; Nishimoto et al., 1995; Steadman et al., 1991) and field studies (Bacanskas et al., 2004; Bainy et al., 1996; Eufemia et al., 1997; Livingstone et al., 1992, 1995; McClain et al., 2003; McFarland et al., 1999; Otto and Moon, 1996; Porte et al., 2002; Rodríguez-Aziza et al., 1992; Stein et al., 1992; Stephensen et al., 2000; van der Oost et al., 1996; Ventura et al., 2002), no single biomarker of oxidative stress has emerged that is as sensitive and specific as other established biochemical biomarkers such as acetylcholinesterase activity for organophosphate insecticides, δ-ALAD activity for exposures to lead, or CYP1A expression/activity for aryl hydrocarbon receptor agonists (see Chapter 16). Various studies have identified GSSG:GSH ratios, levels of MT or lipid peroxidation, and activities of GR, microsomal GST, or microsomal GPX as the most sensitive indicators in the system being studied, but these markers have been completely unresponsive in other contexts. An extensive review of biomarker studies that included a specific review of biomarkers of oxidative stress (van der Oost et al., 2003) failed to identify any marker that was responsive in a high percentage of the studies reviewed, with lipid peroxidation being perhaps the most consistent marker. Representative examples of field studies employing biomarkers of oxidative stress are provided in Table 6.5.

Why have better markers of oxidative stress in wild fish not been identified? A variety of factors may be involved. First of all, as in mammals, large inductions (e.g., comparable to the inductions in CYP1A observed after exposure to certain xenobiotics, as discussed in Chapters 4 and 16) in antioxidant enzymes have not been observed in fish exposed to prooxidants. As a result, any confounding factors present have a strong chance of hiding an otherwise observable effect. Unfortunately, there are many such confounding factors. An environmental pollution mixture will rarely be expected to exert toxicity only by oxidative stress, so other forms of toxicity may be occurring. Additionally, sex and reproductive condition can affect many antioxidant parameters (Livingstone et al., 1995; McFarland et al., 1999; Meyer et al., 2003; Winzer et al., 2001, 2002a,b) but are not always taken into account. In that case, the variance associated with sex becomes "noise," potentially obscuring real differences. Similarly, temperature can greatly alter the metabolic capacity of poikilotherms and has been shown to affect antioxidant defenses in fish (Heise et al., 2003; Olsen et al., 1999; Parihar and Dubey, 1995; Parihar et al., 1996). Diet alters the activity of many antioxidant enzymes (George et al., 2000; Hidalgo et al., 2002; Mourente et al., 2000, 2002; Pascual et al., 2003), as well as altering the tissue concentrations of nonenzymatic antioxidants, as mentioned above. Dissolved oxygen (Cooper et al., 2002; Hermes-Lima and Zenteno-Savín, 2002; Lushchak et al., 2001; Ritola et al., 2002; Ross et al., 2001) and salinity (Kolayli and Keha, 1999; Martínez-Álvarez et al., 2002) have also been observed to affect antioxidant parameters. Seasonal effects have been observed (Bacanskas et al., 2004; Ronisz et al., 1999) and are likely to incorporate many other biological and environmental variables, such as temperature, reproductive status, and food sources. Additionally, time courses can be complicated; for example, although total glutathione levels can increase dramatically in response to prooxidant exposure, the initial response is often depletion, and degree of induction is very likely to be additionally affected by diet, as the availability of cysteine can be limiting for the production of GSH. Antioxidant enzymes have also been observed sometimes to be depressed at the level of activity or expression after exposure to prooxidants (Fujii and Taniguchi, 1999; Kim and Lee, 1997; Pedrajas et al., 1995; Radi and Matkovics, 1988; Stephensen et al., 2002; Zikic et al., 1997). Developmental stage is another variable to be considered (Peters and Livingstone, 1996).

Physiological or genetic adaptation to pollution has been shown to lead to an altered response even in biomarkers that are usually fairly robust, such as CYP1A (Elskus et al., 1999; Hahn 1998; Meyer et al., 2002; Roy et al., 2001; see also Chapter 15). Although the possibility of adaptation to oxidative stress in fish has been less studied, it may also be a significant factor in some cases (Bacanskas et al., 2004; McFarland et al., 1999; Meyer et al., 2003). Markers of damage, such as lipid peroxidation and DNA damage, may only reflect recent or constant damage, as both can be repaired. Another important

TABLE 6.5

Oxidative Stress Associated with Field Exposures

Contaminated Site	Suspect Chemicals	Species	Biomarker Response	Refs.
Sediment from Puget Sound, Washington	PAHs, HAHs	English and rock sole, starry flounder	GSH↑	Nishimoto et al. (1995); Stein et al. (1992)
Contaminated sites in the North Sea, U.K.	PAHs, HAHs, metals	Dab	DT diaphorase, GPx	Livingstone et al. (1992)
Sediment from Black Rock Harbor, Connecticut	PAHs, HAHs	Channel catfish	SOD, catalase, GSSG, GSH, lipid peroxidation↑	Di Giulio et al. (1993)
Sediment from Seaforth Docks, Liverpool	PAHs, HAHs	Dab	SOD, catalase, lipid peroxidation↑	Livingstone et al. (1993)
Duwamish River, Washington	PAHs, HAHs	English sole	Oxidative DNA lesions↑	Malins and Gunselman (1994)
Polluted reservoir, Sao Paolo, Brazil	Domestic and industrial effluent	Nile tilapia	SOD, GPx, G6PDH↑, GSH↓	Bainy et al. (1996)
St. Lawrence River, Ottawa, Canada	Industrial effluent	Brown bullhead catfish	SOD↑, catalase, GPx, GSH↓	Otto and Moon (1996)
Yamaska River, Québec, Canada	Pesticides	White sucker	GPx, catalase, GSH↓, lipid peroxidation↑	Dorval et al. (2005)
Niagara River ecosystem, New York and Canada	PAHs, HAHs, pesticides	Brown bullhead catfish	GSH↑	Eufemia et al. (1997)
Wabush Lake, Newfoundland, Canada	Mine tailings	Lake trout	Oxidative DNA damage↑	Payne et al. (1998)
Cadiz Bay, Spain	PAHs, HAHs, metals	Gilthead sea bream	Oxidative DNA damage↑	Rodriguez-Aziza et al. (1999)
Black River, Ohio	Coking plant (PAHs)	Brown bullhead catfish	SOD↑, catalase and GSH↓	McFarland et al. (1999)
Harbors on the Icelandic coast	Various	Shorthorn sculpin	GR, GPx, and catalase↑	Stephensen et al. (2000)
Morava River Basin, Czech Republic	Organochlorine pesticides, PAHs, HAHs, metals	Chub	GR↑	Machala et al. (2001)
Western Mediterranean coast	Various	Red mullet	Catalase↑	Porte et al. (2002)
Rio de Janeiro coast	Industrial and domestic effluent	Spotted pigfish	Catalase↑	Ventura et al. (2002)
Elizabeth River, Virginia	Former wood treatment plant (PAHs)	Atlantic killifish	GSH, GPx, lipid peroxidation↑	Bacanskas et al. (2004)

Note: Representative biomarker studies were performed on fish from contaminated field sites or on fish exposed in the laboratory to contaminated sediments taken from field sites. Note that in many cases exhaustive chemical analysis was not performed, so the chemicals to which the fish were exposed are not well defined in those cases. Not all biomarker responses observed are listed but only those that fall into the category of oxidative stress and which showed a clear change.

concern is that most fish populations are highly outbred with a high degree of genetic variability (compared to typical laboratory mammalian models), which may contribute additional variability to the responses observed. Finally, as mentioned earlier, the various antioxidant isoenzymes potentially represented in fish are not well characterized, and it may be that potentially good biomarkers are being overlooked due to nonspecific substrates, antibodies, etc. An improved understanding of biological and environmental modulation of oxidative stress and antioxidant defenses in fish, along with careful experimental design and improved techniques, should enhance the utility of biomarkers for oxidative stress in the future; however, as discussed above, no single broadly applicable biomarker for oxidative stress is likely to emerge in the foreseeable future.

Oxidative Stress and the Health of Wild Fish Populations

As described above, oxidative stress is known to play an important role in many disease processes and chemical-mediated toxicity in humans. Furthermore, it is clear that there are many similarities among humans, mammalian models, and fish in terms of production and cellular effects of oxidative stress, as well as in terms of antioxidant defenses; nonetheless, the importance of oxidative stress to fish populations in the wild is not yet fully understood. Biomarkers of effect of exposure to ROS such as lipid peroxidation (Di Giulio et al., 1993; Eufemia et al., 1997; Livingstone et al., 1993) and DNA damage (Di Giulio et al., 1993; Eufemia et al., 1997; Malins and Gunselman, 1994; Payne et al., 1998; Rodríguez-Aziza et al., 1999; Stein et al., 1992; Stephensen et al., 2000; Sugg et al., 1996; Theodorakis et al., 1997, 1999) indicate that fish inhabiting polluted environments exhibit oxidative stress (although not all of these DNA damage studies are necessarily strictly oxidative-stress related). Some common fish diseases, such as jaundice and methemoglobinemia ("brown blood disease") have been associated with oxidative stress (Jensen, 1996; Sakai et al., 1998). Some studies suggest that the highly teratogenic effects of TCDD are related to its ability to produce ROS mediated by the catalytic activity of cytochrome P4501A (Cantrell et al., 1996; Dong et al., 2002; Schlezinger et al., 1999; Teraoka et al., 2003); however, this remains controversial (Carney et al., 2004).

Fish populations adapted to inhabit ecosystems contaminated with chemical mixtures containing prooxidants such as creosote (Meyer and Di Giulio, 2003) and metals (Weis et al., 1999; Xie and Klerks, 2003) have been shown to be less fit than their counterparts from clean sites in several contexts. There are many published examples of fish populations inhabiting sites contaminated with prooxidant chemicals that exhibit high rates of DNA damage and cancer (Black, 1983; Malins and Gunselman, 1994; Malins et al., 1984; Mix, 1986; Vogelbein et al., 1990). Although the chemicals present at such sites are not exclusively toxic via prooxidant mechanisms, a strong relationship exists between oxidative stress and cancer (Klaunig et al., 1998). Furthermore, the ability of channel catfish to avoid cancer in the same polluted environments that cause liver cancer in brown bullhead catfish has been associated with higher antioxidant defenses (Hasspieler et al., 1994a,b; Ploch et al., 1999). Dietary exposure to hydrogen peroxide enhanced the development of oxidative DNA damage (8-OHdG) and hepatic tumors in rainbow trout exposed to the carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in a laboratory study (Kelly et al., 1992). As in the case of human epidemiological studies, however, it is difficult to unequivocally link a given pollutant or form of toxicity with population-level measurements of health. Long-term laboratory exposures will likely be needed to better define the links between exposure to oxidative stress and ecologically relevant alterations in fish populations, and improved markers of oxidative stress will be necessary to analyze the responses of populations of wild fish to oxidative stress.

Future Directions

A better understanding of which antioxidant genes are present in fish, how they are regulated, and what their biochemical characteristics are will greatly improve our ability to understand the impact of oxidative stress and the impact of prooxidant chemicals on fish. Similarly, application of the knowledge gained in studies of oxidative stress in mammalian models will inform improved generation of hypotheses and experimental design; for example, we know now that very large inductions in expression of classical

antioxidant genes such as superoxide dismutase, catalase, or glutathione peroxidase should not be expected either in mammals or in fish. Future studies will be likely to examine the expression of genes shown in mammals to respond strongly to oxidative stress but which have received less attention in fish (e.g., heme oxygenase). Similarly, they may examine additional parameters and subcellular compartments; for example, mitochondria are an important target of oxidative stress (Cadenas and Davies, 2000; Finkel and Holbrook, 2000; Kowaltowski and Vercesi, 1999; Krumschnabel et al., 2005; Outten et al., 2005; Yakes and Van Houten, 1997), but mitochondria or mitochondrial-specific parameters such as MnSOD, mitochondrial glutathione pools, and mitochondrial GPX have received little attention. Molecular characterization of antioxidant enzymes in fish may provide guidance by demonstrating which genes are ARE regulated and which are not. Future studies should also take into account (through experimental design and/or statistical analysis) as much as possible the effects of confounding biological and environmental factors such as sex, season, oxygen tension, and diet. Improved methodology for the measurement of DNA damage, lipid peroxidation, and other biomarkers of effect will permit more sensitive assays, and increased knowledge of fish-specific forms of antioxidant enzymes will provide more specific assays. Further testing of relatively new techniques such as the TOSC assay may lead to powerful new tools. The development of transgenic fish with reporter genes activated by oxidative stress (Carvan et al., 2001) may result in highly specific measurements of the generation on ROS *in vivo*, in the laboratory, or in the field. Assays that take advantage of fish-specific biology may also prove valuable; for example, the presence of nucleated blood cells in fish permits a nonlethal measurement of DNA damage (Tiano et al., 2001; Villarini et al., 1998) as well as other endpoints (Anderson, 1994; Bainy et al., 1996; Gabryelak and Klekot, 1985; Ritola et al., 2002).

At the same time as biochemical and molecular methodologies are improved and mechanistic understanding of oxidative stress in aquatic organisms is refined, it will be important to begin to more carefully explore relationships between exposure to oxidative stress and population-level effects. The associations between contaminant exposure and cancer epizootics and other fitness costs mentioned above suggest that oxidative stress is an ecologically important phenomenon; however, few attempts have been made to quantify the costs of the oxidative damage caused by environmental pollutants for individual fish, fish populations, and aquatic ecosystems. Although a mechanistic understanding of oxidative stress in fish is important from the standpoint of comparative toxicology and basic biology, a solid understanding of the ecological importance of oxidative stress is dependent upon studies of higher level effects.

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Unit II

Key Target Systems and Organismal Effects

7

Liver Toxicity

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CONTENTS

Introduction

A range of contaminants including carcinogens, metals, biotoxins, and persistent organic pollutants injure the livers of fishes, and, although some mechanisms of liver injury are unique to fish, often hepatic injury in these aquatic vertebrates arises from mechanisms similar to those observed in mammals. Development and approval of pharmaceuticals, personal-care products, and cosmetics and the identification of occupational safety risk factors have led to an abundance of information regarding hepatic injury in mammals. For fish, no such large-scale programs have served to further understanding of hepatic toxicology. For students of the toxicology of fishes, it is important to review some of the interests that have led to the current state of our knowledge regarding toxicity in this target organ. Interest in the investigation of liver toxicity and injury in fishes stems from several motivations, including developing an understanding of vertebrate comparative physiology and anatomy; addressing problems in aquaculture associated with liver pathologic conditions caused by nutrition-related factors or improper storage of dietary components; analysis of the pathogenesis of liver neoplasia in selected fish species; and, more recently, development of biomarkers of exposure and effect and use of toxic alterations/responses in risk effects assessment. The latter interest stems from the fact that the aquatic medium is a sink for many anthropogenic contaminants (Long and Buchman, 1990; Mackay, 1992a,b; Tanabe et al., 2004). Pioneering workers recognized the unique strengths emanating from toxicological and biomedical studies comparing the spectrum of vertebrates (Guarino, 1987). Also, within the taxonomic class of fishes, where the largest group of vertebrates are found, comparative approaches can provide important insights, given the numerical, physiological, and ecological diversity herein. Recently, the transfer and incorporation of exciting new molecular technologic advances from the biomedical arena to environmental monitoring and assessment have been realized (Larkin et al., 2003; Rise et al., 2004; Williams et al., 2003). Strengths of fish models are now increasingly recognized and being used by the biomedical community (Barut, 2000; Dodd et al., 2000; Larkin et al., 2003; Loosli et al., 2000). Students of fish liver toxicology will need to achieve integration of various approaches/disciplines to use these new tools effectively and to interpret findings in their model systems. There is a need to push our understanding of fish liver toxicology while maintaining contact with advances in the biomedical arena. We feel that the time is ripe for a critical assessment of the hepatic toxicology of fishes, and we hope that this chapter portrays the progress achieved and stimulates and guides future studies.

The Liver as a Target Organ

In part, the structure and function of the normal liver in fishes make it a target organ for toxic chemicals. To better understand this statement, we must first consider the position of the liver, its afferent and efferent vascular relationships, its microvascular properties that facilitate uptake, and how toxicants

reach this organ. The liver has a dual blood supply. Arterial blood reaches the liver by way of the hepatic artery, a branch of the celiacomesenteric artery from the dorsal aorta. An afferent venous supply, the hepatic portal vein, is the major source of blood to the liver. Whether the source is arterial or venous, blood from both sources enters the capillary bed, hepatic sinusoids, or microvasculature of the liver. In turn, this bed of capillary-like vessels, the hepatic sinusoids, is the basis for the liver being referred to as a richly perfused compartment, one especially equipped with special modifications of the endothelial cells (i.e., fenestrae and an absence of basement membrane) that facilitate rapid uptake of substances. The exit path for hepatic blood is also of great importance, as the release of toxic substances such as acute-phase proteins following liver injury may affect the structure and function of downstream organs (heart, gill, brain). Venous flow from the liver is described later in this chapter.

Due to the dual blood supply of the liver, it is important for us to consider two principal routes of uptake that may lead to hepatic distribution of potentially toxic compounds. These are the intestinal uptake route and the branchial one. For important information in this regard, the reader is referred to two other chapters in this text, Chapter 8 and Chapter 3.

For those compounds with log K_{ow} values of 3 to 4, accumulation by fish is primarily by the aqueous route (McKim and Nichols, 1994), and much of this will involve the gill. These compounds may first interact with the surficial branchial and branchial endothelial cells. From here the compounds may take a more direct route to the liver but one that requires passage into the dorsal aorta and its branches, as described above, before it reaches the liver. For that portion of branchial blood that goes directly to the head or is distributed to organs, such as the swim bladder, an indirect path involving venous return of compounds back to the heart and another pass through the branchial circulation before entry in liver are used. Clearly, the liver is not an initial or primary target for compounds taken up by tissues such as the gills (Barron et al., 1989) but may be exposed to a combination of parent compound and metabolites from more proximate cells and tissues. With the swallowing of water, the gut becomes a possible route for the uptake of polar compounds. With this route, the liver is a more direct target, but metabolism within the wall of the intestine (Van Veld et al., 1997) is likely prior to entry in liver.

For those compounds with log $K_{ow} > 6$, uptake will likely be by the dietary route. This includes polychlorinated biphenyls (PCBs), dioxins, dichlorodiphenyltrichloroethane (DDT) and its metabolites, and other persistent organic pollutants (POPs). For the sake of liver toxicity, important questions include: How much and what type of metabolism occurs prior to entry in liver? On a volume basis, the liver receives the vast majority of its blood supply by way of the hepatic portal vein. This strategic location means that capillary beds within the walls of the stomach (gastric fishes), the entire intestinal tract, and the spleen are tributaries to the hepatic portal vein. The liver is therefore an early but not the first organ to encounter ingested nutrients, vitamins, metals, drugs, and environmental toxicants, as well as waste products of bacteria that enter hepatic portal blood. At the present, we lack information on first-pass metabolism in the wall of the alimentary canal and in the spleen; however, ample evidence from studies with toadfish (*Opsanus tau*), spot (*Leiostomus xanthurus*), channel catfish (*Ictalurus punctatus*), mummichog (*Fundulus heteroclitus*), and tilapia (*Oreochromis mossambicus*) suggests that the intestine, and not the liver, is the initial metabolic organ following dietary exposure to polycyclic aromatic hydrocarbons (PAHs) and halogenated hydrocarbons (James and Kleinow 1994; James et al., 1996, 1997; Kleinow et al., 1998; Van Veld et al., 1987, 1988; Vetter et al., 1985; Yeung et al., 2003). How much of the compound is sequestered in other organs prior to the liver? We are not aware of studies that have shown a differential hepatic toxicity in relation to the uptake route. At the present time, it is difficult to make a reasonable prediction on the relevance of specific uptake routes for liver toxicity.

Finally, the skin may represent an important route for uptake, particularly for the early life stages of fish. From the skin, venous return to the sinus venosus, passage through the heart, and return through the branchial circulation (to a varying degree dependent on development) would be necessary before the compounds would reach the liver. Given the strategic location of the liver, however, its complement of enzymes, its dual blood supply, and the enterohepatic circulation, this organ is a major target for xenobiotics whatever the exposure route considered.

Major Functions of the Liver

The three major functions of the liver essential for life of the organism are:

- *Uptake, metabolism, storage, and redistribution of nutrients and other endogenous molecules*—The synthetic and excretory functions of the liver maintain the homeostasis of the organism. To achieve this, specific molecules are synthesized in hepatocytes, packaged in the Golgi apparatus, transported in a specific direction for release into the intercellular spaces and to the bloodstream, where they are taken up by other organs and utilized. Some examples of storage, synthesis, and redistribution functions include glycogenolysis and hepatocyte release of glucose to govern blood glucose levels; hormone synthesis and release (e.g., somatomedins); synthesis and release of proteins, as in the case of serum albumins, the yolk precursor vitellogenin, and the zona radiata protein or choriogenin. The removal, metabolism, and eventual excretion of compounds also participate in the homeostatic role of the liver; for instance, hormones are taken up and broken down by the liver.
- *Metabolism of lipophilic compounds, including xenobiotics*—Biotransformation reactions catalyze the conversion of endogenous as well as exogenous compounds with poor water solubility to more hydrophilic metabolites that can be readily excreted. With respect to xenobiotics, the majority of hepatic biotransformation reactions may be considered as a detoxification process decreasing toxic body burden by enhancing excretion. However, during the biotransformation process, generation of electrophilic reactive species can lead to interaction with basic cellular constituents such as DNA and proteins. The end result of this process may be disruption of normal cellular function and overt toxicity, including acute forms and chronic states such as carcinogenesis and tumor formation.
- *Formation and excretion of bile*—Bile excretion is important for the elimination of degradation products of endogenous compounds such as heme or steroid hormones, as well as for the elimination of xenobiotics and their metabolites and some metals such as copper and mercury.

All of these hepatic functions—synthesis and redistribution of nutrients and intermediary metabolites, biotransformation, and bile formation—have been shown to be involved not only in physiological states but also in processes leading to alterations in hepatic morphology and physiology. It is this great metabolic capacity of the liver that makes it both a target and an organ of defence. When toxic hits on the target occur, they may lead to alterations or injury in liver structure and function. Because of the multiple physiologic functions of the liver and its considerable plasticity, the liver responds to toxic insults in many different ways; thus, there appears to be no prototype single reaction classification of hepatotoxicity. Rather, a combination of morphologic pattern, functional alteration, and mechanisms is used to classify hepatotoxicants in mammals (Vandenberghe, 1996) and could be used for fishes as well.

The deterioration of hepatic structure and function not only is relevant for the liver itself but may also lead to aberrations in other organs and to death of the organism. Loss of biliary function alone is incompatible with life. Hepatic clearance function and dysfunction involving the microvasculature and the intrahepatic biliary system for excretion of compounds as well as the pronounced metabolic capacity of this organ may all play a role in the spectrum of toxic conditions possible in this organ. The liver also possesses a pronounced capacity to acclimate to toxic stress, producing protective molecules or performing efficient repair and full or partial recovery. The latter may be considered acclimation, providing for survival of the altered host.

An understanding of chemical hepatotoxicity requires an appreciation of anatomic and physiologic features of the liver. With respect to fish liver, it is important to emphasize that, although fish and mammalian liver agree in many features, there are still differences that may influence chemical toxicity and especially its interpretation. Also, although there are basic similarities of structure and function common to the fraction of the total fish species that have been investigated, species differences must always be considered, and this should involve considerations at all levels of biological organization.

In this chapter, we seek to: (1) briefly review key aspects of liver morphology and physiology of fish, emphasizing those features that differ among fish species and between fish and their mammalian counterparts, and emphasizing why and how these features are relevant to understanding the liver toxicity of fish; (2) review the literature on liver pathology in toxicant-exposed fish and provide insight into major pathological and pathophysiological response patterns of fish liver; and (3) identify specific information gaps and provide suggestions for future directions.

Liver Structure

Fundamental aspects of fish liver anatomy, especially those important in the interpretation of toxicity, were recently reviewed (Hinton and Couch, 1998; Hinton et al., 2001), and the interested reader is referred to these detailed descriptions. In an extensive treatise on the biology and pathobiology of the human liver by Arias et al. (1988), six specific principles were listed that are required and govern structure and function of a liver. We paraphrase these as follows: (1) exocrine and metabolic functions expressed in the same cell (the hepatocyte); (2) a double blood supply (arterial from the dorsal aorta and afferent venous from the hepatic portal vein); (3) a specific architectural arrangement of single cells and cell masses that facilitates exchange between blood and hepatocytes; (4) features of perihepatocellular or perisinusoidal spaces (of Disse), such as the absence of basement membrane, thereby maximizing the exchange between blood and hepatocyte; (5) separation of the lumen of biliary from blood spaces, thereby compartmentalizing excretory from other hepatocytic functions; and (6) specific biochemical activities of hepatocytic receptors, enzymes, organelles, and cell membranes that regulate and enable specific functions.

As is demonstrated in subsequent sections of this chapter, fish hepatocytes fulfill requirement 1 above. In addition, we review and provide new evidence for the dual blood supply (requirement 2). The specific architectural pattern of cell masses is very similar in embryos and larval equivalent livers of mammals and fishes; however, with respect to requirement 3, mammalian adult liver differs from livers of fishes, reptiles, amphibians, and birds. Electron microscopic observations reveal fenestrae in sinusoidal endothelium of mammals and fishes, and both lack a basement membrane to facilitate exchange (requirement 4). Fish liver meets requirement 5, and these functional characteristics are reviewed in the subsequent section.

Despite of the many structural similarities between the livers of fishes and those of mammals, certain features of hepatic gross and microscopic anatomy of fishes are recognized as different (Hampton et al., 1985, 1988, 1989). Some of these include the number of lobes; presence or absence of lobules and portal tracts; architectural arrangement of hepatocytes; the absence in many fish species of Kupffer cells; and the distinctive and common presence of macrophage aggregates in fishes (Table 7.1).

Coverage of liver anatomy in fish is not complete without mention of the exocrine pancreatic cells that occupy the periadventitia of portal veins within the body of the liver. Certain species contain abundant pancreatic cells (varying in amount), thus leading to the term *hepatopancreas*. The recent observations and three-dimensional reconstructions of the tilapia (*Oreochromis niloticus*) should be consulted (Figueiredo-Fernandes et al., 2007).

The above structural similarities and differences represent more than a simple academic exercise in comparative liver anatomy; rather, they are important in interpretation of toxic responses within livers of fishes. *In vitro* studies with isolated primary hepatocyte preparations (Rabergh and Lipsky, 1997) or precision slices of trout liver (Singh et al., 1996) have clearly shown that exposure is followed by the presence of cytosolic enzymes in culture medium, validating the toxic nature of these responses. Architectural (the absence of lobules) and physiological (lack of metabolic zonation; see below) differences in fish vs. rodent livers apparently lead to different histological patterns of toxicity. Assuming that the absence of a mosaic pattern of response, such as centrolobular necrosis following carbon tetrachloride exposure of rodents (see below), indicates that fish are not capable of responding to such agents is erroneous; instead, other patterns of morphologic response are likely in fish (Droy, 1988).

TABLE 7.1

Comparison of Rodent and Fish Hepatic Anatomical Features

Feature	Rodent	Fish
Liver lobes	Multiple	Usually single; cyprinids are exception
Lobules	Distinct	Indistinct or absent; implies different arrangement
Portal tracts	Classic lobule contains bile duct, portal venule, and hepatic arteriole and defines sites where arterial blood enters hepatic microcirculation	Absent; larger bile ducts may coexist with hepatic artery in so-called "biliary arterial tract"; no portal vein branches are in biliary arterial tracts
Blood supply	Dual supply by hepatic artery and hepatic portal vein (major volume); capillary-like sinusoids contain arterial and afferent venous blood	Dual supply by hepatic artery and hepatic portal vein (major volume); capillary-like sinusoids contain arterial and afferent venous blood
Venous drainage	Hepatic veins to caudal vena cava	Hepatic veins to sinus venosus
Architecture of parenchyma	Laminae of hepatocytes predominantly one-cell thick and usually separated by sinusoid from nearest neighbor; no biliary epithelial cells in parenchymal compartment	Hepatic tubules comprised of five to eight hepatocytes clustered about a lumen (biliary passageway); parenchyma often contain biliary epithelial cells (see below)
Biliary system	Canaliculi sole component in lobules, with network extending from center of lobule to periphery; cholangioles (ducts of Hering) at lobular margin; small bile ducts in portal tracts; large intrahepatic ducts near hilus	Canaliculi formed by lateral plasma membranes of adjacent hepatocytes; after short course communicate with lumen of hepatic tubule; transitional biliary epithelial cells and cholangioles present at center of tubules
Kupffer cells	Present	Generally absent; *Ameiurus* species are exceptions
Perisinusoidal macrophages	Present	Present
Macrophage aggregates	Absent	Present

In early embryonic stages, human hepatocytes form cords or tubules in which (as in a gland) multiple epithelial cells appear on the cut surface to surround the lumen. The epithelial cell masses cluster around a biliary vascular axis to which afferent blood is brought from hepatic arteries and portal veins, and bile is secreted in the opposite direction (Arias et al., 1988). As metabolic demands rise for mammals, the gland-like cords are transformed into a sponge-like wall work, or cellular mass, which is composed of hepatocellular plates (or laminae) that are predominantly one hepatocyte thick; they meet at different angles and surround the hepatic lacunae (microvasculature and spaces immediately surrounding the capillary such as sinusoids). In the human, metamorphosis from cord or tubule to laminae of hepatocytes is completed by age 5 (Arias et al., 1988). In contrast, the livers of amphibians, birds, fishes, and reptiles retain the tubular architectural pattern as the adult phenotype (Hampton et al., 1985; Hinton and Couch, 1998; Hinton et al., 2001). This glandular arrangement of cells within the piscine liver translates into an abundance of biliary epithelial cells in close proximity to hepatocytes within the parenchymal compartment (Hinton and Couch, 1998).

Gross inspection of the livers of fish reveals that most are in the form of a single lobe (Figure 7.1), whereas livers of mammals have multiple lobes. Cyprinid fishes such as *Cyprinus carpio* and *Danio rerio* have extensions from the major liver mass that extend along loops of the intestine (Amlacher, 1954). These may be regarded as multiple lobes (Figure 7.2). The relationships of the liver to neighboring organs are shown in Figure 7.3 and Figure 7.4.

The classic lobule, a consistent microanatomical feature of mammalian liver, is not present in the livers of fish, as has been illustrated in high-resolution light micrographs from medaka liver (Figure 7.5). Mammalian portal tracts, found at corners of the classic lobule, are variably delimited in different mammalian species by perilobular connective tissue containing the bile duct, portal venule, and hepatic

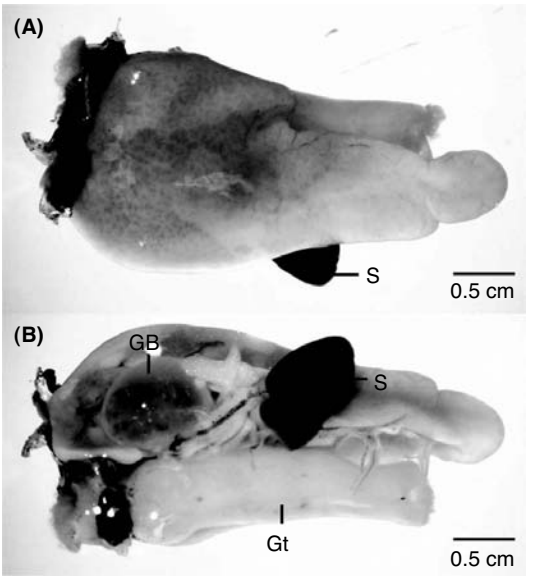

FIGURE 7.1 (See color insert following page 492.) (A) Ventral view of freshly dissected liver of *Fundulus heteroclitus*. This liver is typical of the compact, single-lobed livers of many teleosts. (B) Dorsal view of the liver shown in A. Between the gallbladder and larger spleen, the hilus of the liver is seen. It is here that the hepatic ducts leave the liver and the arterial and afferent venous blood supplies enter the organ. GB, gallbladder; Gt, gut; S, spleen.

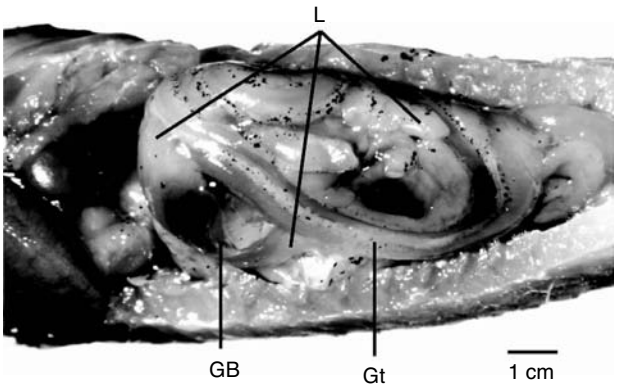

FIGURE 7.2 (See color insert following page 492.) Freshly dissected visceral mass from goldfish (*Cyprinus carpio*) illustrating the liver pattern of some teleosts in which the organ is comprised of multiple lobes or extensions between coils of the intestine. The black material is due to melanocytes in the visceral peritoneum of this abdominal cavity. Establishing a precise liver weight or dissecting the organ for biochemical or molecular analyses would prove difficult with livers of this form. Arrows point to portions of the liver in close proximity to the elongated intestinal tract. GB, gallbladder; Gt, gut; L, liver.

arteriole (with a lymphatic vessel and a nerve). Portal tracts are anatomic indications of sites where arterial and afferent venous blood enters the parenchymal compartment (lobule). Hepatocytes adjacent to portal tracts receive more oxygenated blood than do those cells at central portions of lobules. This lobular zonation of mammals (Sasse et al., 1979) is important when interpreting toxic responses and classifying various hepatotoxicants when exposure results in a mosaic pattern of altered and unaltered parenchyma, depending on the targeting of afferent or efferent zones by the toxicant. No such anatomical mosaic pattern has been observed in fish exposed to reference hepatotoxicants (see section on responses of fishes to reference hepatoxicants, below). The heterogeneity of mammalian hepatocytes has been demonstrated with regard to oxygen saturation, cell volume, glycogen, isoforms of cytochrome P450 and other enzymes, and cell shape. This has led to the concept of metabolic zonation within the

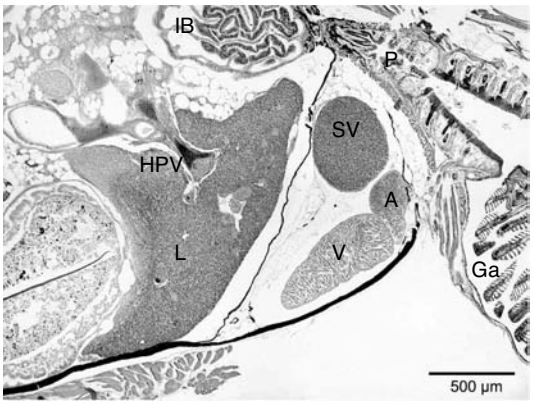

FIGURE 7.3 (See color insert following page 492.) Hematoxylin and eosin stain of section through adult medaka (*Oryzias latipes*). Rostral structures are oriented toward the right side of the field; dorsal is top and ventral bottom. Parasaggital section shows portions of the abdominal cavity, pericardial cavity, branchial chamber, and pharynx. A, entry to aorta; Ga, gill arch with primary lamellae attached; HPV, hepatic portal vein in liver hilus; IB, intestinal bulb; L, liver; P, pharyngeal mucosa with teeth; SV, sinus venosus; V, ventricle. See micron bar for magnification.

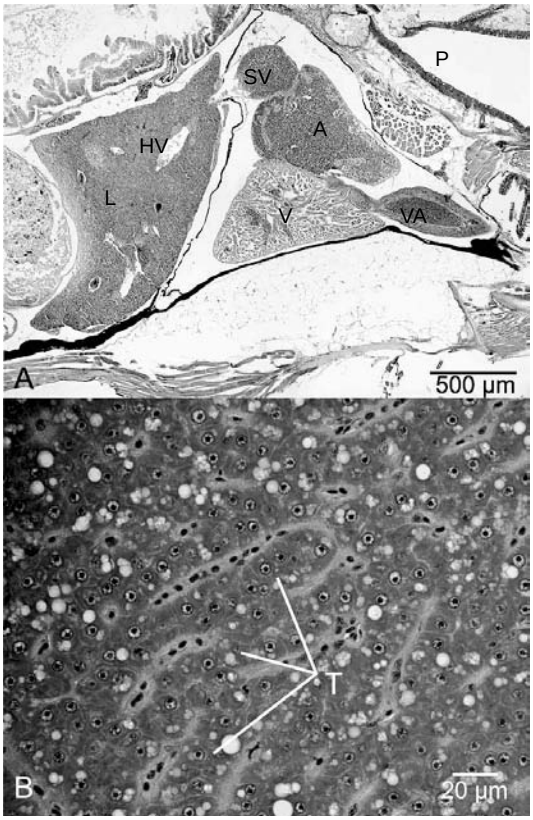

FIGURE 7.4 (See color insert following page 492.) (A) Stain and orientation are identical to Figure 7.3. A, atrium; HV, hepatic vein conducting blood from liver to sinus venosus (SV); L, liver; P, pharynx; V, ventricle; VA, ventral aorta. Melanin is in parietal pericardium and parietal peritoneum. See micron bar for magnification. (B). Section of adult female liver shows basophilic cytoplasm overlying endoplasmic reticulum (purple areas of hepatocytes). Large vacuoles are fat. This appearance is that of the reproductively active female with vitellogenin production. T, hepatic tubule formations.

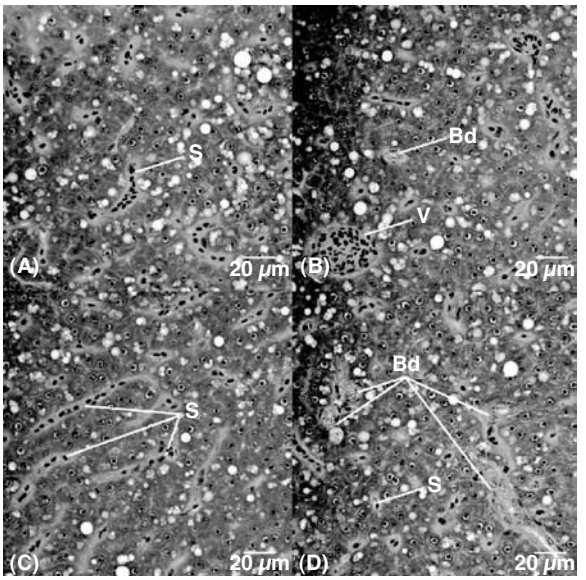

FIGURE 7.5 (See color insert following page 492.) High-resolution light micrographs of medaka liver showing elements of hepatic architecture. (A) Hepatic tubules comprised of hepatocytes and biliary epithelial cells are partially separated by sinusoids (S). No lobular architecture is apparent. Rounded white structures are lipid vacuoles. Lipid was removed during alcohol dehydration in processing. (B) Hepatic sinusoids contain nucleated red blood cells (top right of field). Larger venule (V) has no arterial or biliary structure associated. (C) This field shows sinusoids (S) between which are found hepatic tubules in longitudinal array. Note the double row of hepatocytes making a single tubule. Tubules are incompletely separated by sinusoids. (D) Region near the hilus of the liver is shown. Intrahepatic bile ducts of varying sizes (Bd) are shown. Note the difference in staining and in nuclear profiles in biliary epithelial cells vesus hepatocytes.

mammalian liver lobule (Jungermann, 1995; Osypiw et al., 1994); however, investigators studying the livers of teleosts report less (Schar et al., 1985), little (Segner and Braunbeck, 1988), or no (Hampton et al., 1985) evidence for hepatic metabolic zonation (see section on heterogeneity in fish liver metabolism, below).

Cells of the Liver

Detailed light and electron microscopic studies of fish liver have shown that the morphology of this organ includes at least ten resident cell types (Lester et al., 1993). By far the most numerous, hepatocytes occupy about 80 to 85% of the liver volume and represent about 95% of the resident cell number (Hampton et al., 1989). Together with bile preductular epithelial cells (BPDECs) they form hepatic tubules (see review in Hinton and Couch, 1998). Other epithelial cell types include biliary epithelial cells of ductules and intrahepatic ducts located near the liver hilus, together comprising the intrahepatic biliary system (explained in greater detail below); exocrine pancreatic cells; and centroacinar and ductular cells of exocrine pancreas. These cells share a common embryonic origin: endodermal derivatives from embryonic foregut epithelium (Elias and Bengelsdorf, 1952). The remaining cells are mesodermal in origin, arising from yolk sac epithelium, primitive blood islands, and septum transversum. These mesodermal derivatives include hepatic stellate cells, endothelium and smooth muscle of blood vessels, macrophages, and fibroblasts.

Hepatocytes

Hepatocytes are the most numerous cell type in the hepatic parenchyma, and they occupy greater than 80% of the liver volume in trout (Hampton et al., 1989; Rocha et al., 1994). Surface modifications of the hepatocyte plasma membrane form canaliculi (Figure 7.6), the initial portion of the hierarchy

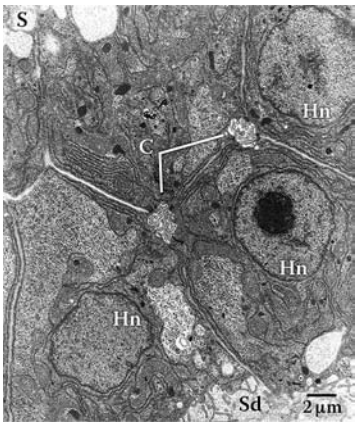

FIGURE 7.6 Transmission electron micrograph of liver from larval medaka. Portions of six hepatocytes are shown. The space of Disse (Sd) is at the bottom right and top left corners. Here, hepatocytes extend finger-like spaces toward sinusoids. This field shows no stellate cells of Ito in the space of Disse. Hepatocyte plasma membranes form junctional complexes that form walls of bile canaliculi (C). In these spaces, numerous microvilli from hepatocytes fill portions of the lumen. Hn, hepatocyte nucleus; S, sinusoid. The bodies of high electron density in the top-center hepatocyte are lysosomes.

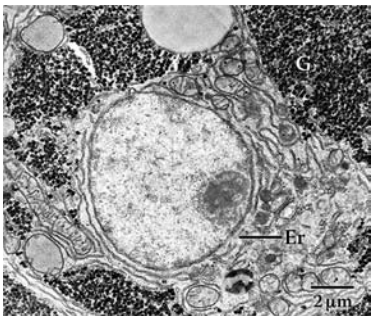

FIGURE 7.7 Area of hepatocyte from common carp with nucleus and surrounding cytoplasm showing polarity. The perinuclear area is abundant in endoplasmic reticulum (Er) and also shows numerous mitochondria. More peripheral areas of the cell are sites of large glycogen depots (G).

of biliary passageways in the liver. Other surface modifications, such as the finger-like projections into the perisinusoidal space of Disse (Figure 7.6), increase the absorptive area for nutritional storage functions of the liver. Hepatocyte nuclei are spherical and are surrounded by a perinuclear sheath of granular endoplasmic reticulum (Figure 7.7). Mitochondria, lysosomes, and peroxisomes are abundant. Cisternae of endoplasmic reticulum (ER) are the sites of various transport lipids. Usually, a prominent Golgi apparatus communicates with ER-derived vesicles. The Golgi apparatus is important in the modifications of ER-derived vesicles that make it possible for them to fuse with plasma membrane, thus enabling exocytosis of contents. The hepatocyte is an example of a single cell serving both endocrine and exocrine functions. Hepatocytes of reproductively mature female fish contain large arrays of granular ER and mitochondria that facilitate their synthesis and the transport of vitellogenin. In contrast, adult males contain large glycogen depots (Figure 7.7) in the peripheral region of hepatocytes. This difference makes it possible to differentiate sexually active females from males by their basophilic hepatocytes seen in standard hematoxylin and eosin stains of paraffin embedded liver. Fish hepatocytes are polar, with sinusoidal plasma membrane representing the basal and canalicular plasma membrane representing the apical aspect. Lysosomes are more numerous in the pericanalicular regions (Hampton et al., 1985, 1988). Table 7.2 provides additional information on the regional distribution of organelles in hepatocytes of rat and carp.

TABLE 7.2

Intrahepatocyte Organelle Distribution

Zone	Rat, Standard Diet[a]		Rat, Fructose Diet[a]		Carp, Standard Diet[b]	
	Mitochondria (%)	Endoplasmic Reticulum (%)	Mitochondria (%)	Endoplasmic Reticulum (%)	Mitochondria (%)	Endoplasmic Reticulum (%)
A	45	54	49	77	68	71
B	28	23	21	12	15	10
C	27	23	30	11	17	19

[a] Data from Riede, U.N. and Sasse, D., *Cell Tissue Res*., 221(1), 209–220, 1981.

[b] Data from Senger (unpublished).

Note: The numbers indicate organelle distribution along a virtual axis from the cell nucleus to the cell periphery. The axis was subdivided into three zones: a perinuclear zone (A), a middle zone (B), and a peripheral zone (C).

Nonhepatocyte Cells of the Liver

It is important to note that liver toxicity is not always the straightforward consequence of toxic impact on hepatocytes, as nonhepatocytes or interactions between hepatocytes and other liver cells can also be key players in the toxic response of the organ.

Biliary Epithelial Cells and the Intrahepatic Biliary System

The biliary system begins with the hepatocytes that are responsible for the uptake of xenobiotics and potentially toxic byproducts of metabolism. Briefly, hepatocytes form water-soluble conjugates that are transported across the plasma membrane into specialized passageways, the canaliculi (Figure 7.6), which are formed solely by junctional complexes between plasma membranes of hepatocytes. The canalicular lumen is delimited by tight junctional complexes (between the membranes of mammalian hepatocytes) that permit the paracellular exchange of solutes (ions and salts) between blood plasma and the canalicular lumen (Arias et al., 1988). Normal lumina of fish canaliculi (like their mammalian counterparts) are nearly completely filled by hepatocyte microvillar processes (Braunbeck et al., 1992; Hampton et al., 1985; Rocha et al., 1997). Intrahepatic bile passageways (IHBPs) are comprised of bile canaliculi (see Figure 7.6 and Figure 7.8) and bile ductules (cholangioles). In addition, morphological studies of normal

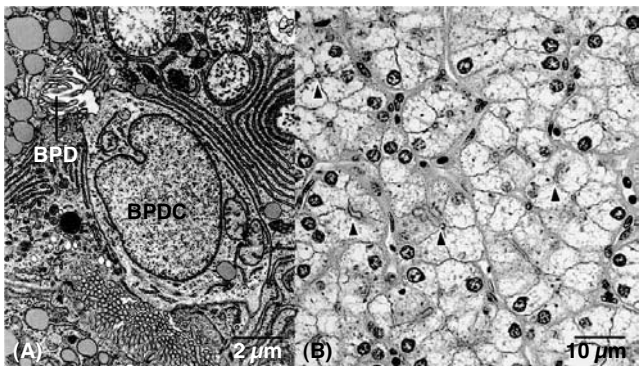

FIGURE 7.8 (See color insert following page 492.) Canaliculi and transitional zones of intrahepatic biliary system are illustrated. (A) Transmission electron micrograph of liver shows portions of two hepatocytes and one transitional biliary epithelial cell, termed bile preductular epithelial cell (BPDC). This cell shares junctional complexes with hepatocytes and forms portion of wall of bile passageway now termed bile preductule (BPD; a transitional zone). Note the absence of microvilli supplied to bile passageway by the BPDC. (B) Light micrograph of paraffin-embedded sturgeon liver. Hepatocytes of this species contain little stainable material in cytoplasm with H&E stain. Note the eosinophilic margins of the cells at bile canaliculi and their extensions toward tubule lumen (black arrowheads). We seldom see bile canaliculi staining this well in other species.

liver from trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) have revealed numerous transitional passageways between the canaliculi of hepatocytes and biliary ductules (Hampton et al., 1988). Transitional passageways (Figure 7.8A), the bile preductules (Hinton and Pool, 1976), are sites where hepatocytes and biliary epithelial cells with oval perikarya share junctional complexes (Hampton et al., 1988). This terminology in fish was adopted based on the flow of bile and the finding of transitional cells in rodent liver between epithelial cells of cholangioles and hepatocytes (Steiner and Carruthers, 1961). Biliary epithelial cells between canaliculi and ductules or cholangioles (completely lined by biliary epithelial cells) were designated in fish as bile preductular epithelial cells (BPDECs) (Figure 7.8). These small, oval cells form junctional complexes with both hepatocytes and adjacent bile ductular epithelial cells. The fine structure of these cells includes scant cytoplasm, intermediate filaments, and an absence of a basal lamina (Hampton et al., 1988). Hampton et al. (1988) extended their observations to bile ductules and ducts in control rainbow trout. As the diameters of bile preductules enlarge, additional biliary epithelial cells contribute to the channel wall (Figure 7.9).

The transition from bile preductules to ductules occurs when the biliary lumen is completely surrounded by biliary epithelial cells, usually two or three, joined by junctional complexes. Lumens of ductules are usually patent and contain few to no microvilli. Some structural variances in canalicular structure have been observed among various teleost species. Cyprinid fishes, for example, have finger-like indentations of the plasma membrane (not shown in the figures) that extend into the hepatocytes and show continuity with the typical interhepatocellular canaliculi (Vogt and Segner, 1997). Mammalian biliary epithelial cells (BECs), or cholangiocytes, are known to play a major role in bile synthesis and secretion. Although BECs account for 3 to 5% of the total population of liver cells in rodents, they are estimated to produce up to 40% of the daily bile output (species dependent) while also modifying bile content (organic or inorganic constituents) through various reabsorptive mechanisms (Boyer, 1996; Nathanson and Boyer, 1991). Few quantitative studies exist for fishes, although biliary epithelial cells in two species of trout comprised 1.3% and 1.4% of the parenchymal volume (Hampton et al., 1989; Rocha et al., 1997) and 3.1% of parenchymal volume in the cyprinid golden ide (Table 7.3). In rainbow trout, when magnesium-dependent adenosine triphosphatase (ATPase) histochemistry is performed, it is apparent that bile ductules receive preductules and canaliculi directly. In addition, scanning electron microscopy, performed on freeze-fractured liver pieces, has shown both the centrotubular location of bile ductules and the connections of canaliculi to these structures (Hampton et al., 1988). Cuboidal biliary epithelial cells rest on basal lamina and line ductules. Near the hilus of the liver, large bile ducts lined by columnar epithelial cells are seen in older trout. The association of macrophages with bile ductules in these control trout suggests that leakage of bile might occur at these sites (Rocha et al., 1994). Collectively, biliary epithelial cells are the second most abundant cell type in teleost liver (Hampton et al., 1989). Examples of the above intrahepatic bile passageways are shown in Figure 7.5D.

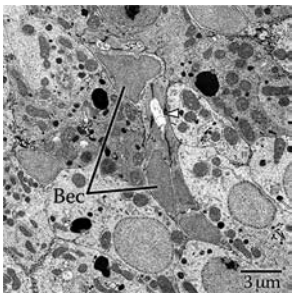

FIGURE 7.9 Transmission electron micrograph of bile ductule. White arrowhead points to the ductule lumen. The ductule is completely surrounded by squamous to cuboidal biliary epithelial cells. Note the relative absence of microvilli in the ductule lumen.

It is often important to be able to differentiate biliary epithelial cells from their neighbors in the liver. To do this, a variety of approaches have proven useful. The circumferential arrangement of ductular and ductal epithelial cells may be visible in sections. Also, a variety of chemical markers for these cells have been reviewed (Hinton, 1993a). The enzyme histochemical reaction for alkaline phosphatase is particularly strong in the connective tissue sheath of medium-sized and larger intrahepatic bile ducts. Biliary epithelial cells are the single resident liver cell type in which gamma-glutamyltranspeptidase is normally found. Magnesium-dependent ATPase is particularly strongly reactive over ductular and ductal epithelium; other enzymes (e.g., glucose-6-phosphate dehydrogenase, diphosphate glucuronosyl dehydrogenase, and DT diaphorase) also mark biliary epithelial cells (Hinton, 1993b). The plasma membrane of biliary epithelial cells is usually positive with the periodic acid Schiff (PAS) reagent, and the mucous granules of tall columnar biliary epithelial cells stain positively. Biliary epithelial cells are positive for cytochrome P450 when immunohistochemical procedures using anti-P450 LM2 IgG, anti-P 450 LM4

TABLE 7.3

Volume Densities of Cells and Spaces in Liver Parenchyma

Component	Rat[a]	Dog[b]	Trout[c,e]	Golden Ide[d]
Hepatocytes	77.8	88.4	84.5 (87.3)	88.9
Nonhepatocytes:	22.2	15.6	15.5 (12.3)	11.1
Endothelial cells	2.8	—	1.8 (1.4)	1.0
Fat-storing cells	1.4	—	0.7 (0.4)	0.1
Kupffer cells/macrophages	2.1	7.8	0.2 (0.7)	0.1
Bile epithelial cells	—	—	1.3 (1.4)	3.1
Spaces:				
Sinusoidal lumen	10.6	4.3	9.4 (6.2)	5.6
Space of Disse	4.9	3.0	1.0 (1.97)	0.5
Bile canaliculi	0.4	0.5	1.1 (0.2)	0.7

[a] Data from Blouin, A., in *Kupffer Cells and Other Liver Sinusoidal Cells*, Wisse, E. and Knook, D., Eds., Elsevier, Amsterdam, pp. 61–70.

[b] Data from Hess, F. et al., *Virch. Arch. Abt. B Zellpathol.*, 12, 303–317, 1973.

[c] Data from Hampton, J.A. et al., *Am. J. Anat.*, 185(1), 58–73, 1989; Rocha, E. et al., *Anat. Rec.*, 247(March), 317–328, 1997.

[d] Data from Segner (unpublished).

[e] Numbers in parentheses are averages of male and female values.

Note: All values are given as percent volume; reference volume = liver parenchyma; volumes were determined by means of stereology.

IgG, and anti-CYP1A are carried out. Ultrastructural localization of the latter antibody, directed against scup CYP450E (Park et al., 1986) and used in conjunction with immunogold procedures (Lester et al., 1993), confirmed the light microscopic observations. In addition, a method to isolate and characterize biliary epithelial cells from trout liver was developed (Blair et al., 1995), and western blot analysis of cytokeratins from trout hepatocytes and biliary cell preparations proved very useful. A significant observation was that hepatocyte preparations exhibited strong immunostaining of a major 52-kDa cytokeratin band with the anti-cytokeratin AE1/AE3, despite the fact that intact hepatocytes (liver sections) did not stain with this antibody. Enrichment of a 55-kDa cytokeratin band in biliary cell preparations compared to starting liver extract indicated a major enrichment of biliary cells by this isolation procedure. A major difficulty in obtaining large amounts of highly enriched populations of biliary epithelial cells from the livers of rats and humans has been their low abundance compared to other cell types of the normal liver. As a consequence, workers have had to rely on conditions that enhance the abundance of biliary epithelial cells. These include bile duct ligation or exposure to toxic chemicals. One advantage of the trout and likely other livers of other fish as a source of biliary epithelial cells is their relatively high abundance under normal circumstances. A ratio of one biliary epithelial cell to every seven hepatocytes has been estimated based on morphometric procedures (Hampton et al., 1989).

Putative Stem Cells of Teleost Liver

The intrahepatic progenitor cells, which are also known as oval cells, and ductular cells of mammalian liver are located at the periphery of the hepatic lobule where they connect with bile canaliculi and extend by canals of Hering into the portal tract. Cells of trout, medaka, and channel catfish found in the intrahepatic biliary system (see above) were shown to form junctional complexes with hepatocytes and to form transitional passageways between canaliculi and biliary ductules (Steiner and Carruthers, 1961), known as bile preductular epithelial cells (BPDECs). These cells of fish share many histological and ultrastructural features with those described for mammalian oval cells (Evarts et al., 1987, 1989; Farber, 1956), and the latter have been shown to proliferate after exposure of rodents to known hepatocarcinogens. A review by Okihiro and Hinton (1999) described the conditions under which oval cells may transition into hepatocytes (Evarts et al., 1989) or biliary epithelial cells (McLean and Rees, 1958; Steiner and Carruthers 1962, 1963). Furthermore, the presence of shared phenotypic markers among hepatocytes, oval cells, and biliary cells, as well as the appearance of transitional phenotypes, has prompted many

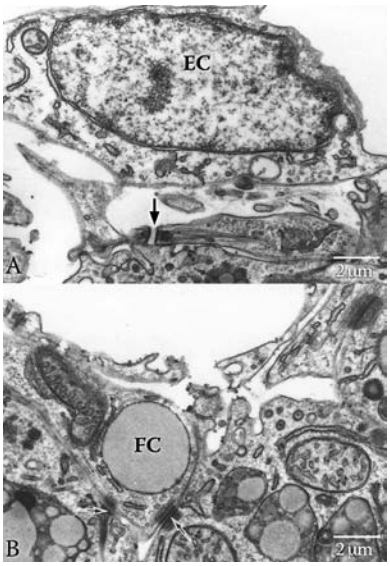

FIGURE 7.10 (A and B). These transmission electron micrographs illustrate the fine structure of sinusoidal endothelial cells and the space of Disse containing portions of stellate, fat-storing cells of Ito. (A) Nucleus of endothelial cell (EC) and perikaryon showing sparse mitochondria, pinocytotic vesicles, and few profiles of endoplasmic reticulum. Beneath the endothelial cell, the arrow points to the junctional complex between two stellate cells. The hepatocyte is at the bottom of the field. Stellate cells contain extensive cytofilaments and form a skeletal framework in some conditions (see text). (B) This figure shows a round lipid vacuole (FC) in a stellate cell. The stellate cell shares junctional complexes with hepatocytes, which show evidence of steatosis.

researchers to support the hypothesis that oval cells are bipolar progenitor cells for both hepatocytes and biliary epithelium in mammalian liver. Results of partial hepatectomy and bile duct ligation in trout (*Oncorhynchus mykiss*) supported the contention that BPDECs of fish are morphologically, enzymatically, and immunohistochemically similar to mammalian oval cells (Okihiro and Hinton, 2000). Our data are also consistent with studies of trout hepatic cells in long-term primary cultures (Ostrander et al., 1995). The longest lived cells in culture were morphologically similar to BPDECs and had cytokeratin profiles nearly identical to BPDECs in the *in vivo* trout study (Okihiro and Hinton, 2000). The maintenance of presumptive BPDECs (up to 70 days) in culture is indicative of relatively immature (i.e., undifferentiated) cells that retain the ability to divide and to survive and is consistent with a stem cell or progenitor role. BPDECs of fish liver appear to be the teleost equivalent of bipolar hepatic stem cells.

Sinusoidal Endothelial Cells

Endothelial cells comprise the wall of the hepatic microvasculature and are therefore the most abundant of the liver endothelial cells (Figure 7.10). Abundant information is available from various species (Ferri and Sesso, 1981a; Fujita et al., 1980; Gingerich 1982; Hacking et al., 1978; Hinton and Pool, 1976; Langer 1979; Nopanitaya et al., 1979a,b; Tanuma and Ito, 1980; Tanuma et al., 1982) to characterize these cells. Endothelial cells of hepatic sinusoids in channel catfish have greatly attenuated cytoplasmic processes forming a thin barrier between the sinusoidal lumen and the perisinusoidal space of Disse (Hinton and Pool, 1976). The endothelial cell cytoplasm contains numerous free ribosomes, sparse granular ER, abundant pinocytotic vesicles (Figure 7.10), and fenestrae of variable diameters (Hinton and Pool, 1976; Hacking et al., 1978; Langer 1979). Lysosomal abundance differs, as they are rarely seen in some species (Hacking et al., 1978; Tanuma and Ito, 1980) and are more common in others (Ferri and Sesso, 1981a; Tanuma et al., 1982).

Fenestrae have received considerable attention. These structures facilitate the passage of macromolecules from the sinusoidal lumen to the perisinusoidal space of Disse. Freeze-etch replicas (Ferri and Sesso, 1981a; Nopanitaya et al., 1979a; Tanuma et al., 1982) and scanning electron microscopy of liver

following vascular perfusion fixation (McCuskey et al., 1986) were used to study fenestrae. In goldfish (*Cyprinus carpio*), 15 to 20 fenestrae were grouped as sieve plates, like their mammalian counterparts (Wisse, 1972). Trout fenestrae were not as tightly grouped as those of carp. When their diameters were estimated, they ranged from 75 to 150 nm (McCuskey et al., 1986).

Sinusoidal endothelium has proved to be particularly reactive for alkaline phosphatase in enzyme histochemical preparations. In addition, the antibodies directed against the cytochrome P450 isozymes LM2, LM4, and CYP1A label endothelial cells (Lorenzana et al., 1989). Location immediately adjacent to blood cells and the presence of attenuated cytoplasm helps to differentiate endothelial cells from neighboring cells; for example, the strong response with alkaline phosphatase assists in differentiating endothelial cells from hepatic stellate cells (Hinton, 1993b). Little attention has been given in fish to differentiation of venous from arterial endothelium of liver.

Hepatic Stellate Cells

The morphology and functional properties of Stellate cells in various vertebrates have been reviewed (Hinton, 1993b; Wake 1980). The connective tissue cells of the teleost liver include hepatic stellate cells (Ito and Nemoto, 1952), fibroblasts associated with the hilus of the liver, and cells incorporated as a sheath around major blood vessels and bile ducts and located immediately beneath the liver capsule and the blood cells. These cells (see Figure 7.10) and the fibroblasts provide a supportive framework for the liver. Hepatic stellate cells contain an elongated and frequently indented (by lipid droplets, the solvent of vitamin A) nucleus with peripheral heterochromatin. Well-developed granular endoplasmic reticulum with dilated cisternae also characterizes these cells (Rocha et al., 1997). In one significant review, livers from 48 species of fishes were investigated, and fat-storing cells were reported in all but three species (Ito et al., 1962). Since then, two studies (Hinton et al., 1984b; Lauren et al., 1990) have demonstrated the presence of fat-storing cells in medaka (*Oryzias latipes*), one of the three species originally reported to lack them. Another variant of the hepatic stellate cell is devoid of lipid droplets (Nopanitaya et al., 1979a). These cells are fibroblast-like cells with well-developed granular endoplasmic reticulum (Ito and Shibasaki, 1968). First described in human adults and embryos, the cells have been described in various fishes and reptiles. In teleosts, injection of vitamin A causes the number of fibroblast-like cells to decrease while the number of lipid-laden cells increases (Fujita et al., 1980; Takahashi et al., 1978). Perhaps the heaviest accumulation of lipids is found in the Antarctic fish *Dissostichus mawsoni* (Eastman and deVries, 1981). Numerous desmosomal junctions between hepatic stellate cells and other liver cells suggest a mechanical, supporting role for the cells. Numerous cytofilaments are also distinguishing features of these cells. If the cytofilaments are contractile, as has been suggested, the orientation of the hepatic stellate cells may have a role in the regulation of blood flow within the sinusoid. The contribution of these cells to the volume of the parenchyma was estimated for trout (Hampton et al., 1989); they occupied 1.37% of the parenchymal space of females, and the male hepatic stellate cell compartment was about half that of females. Actual volume may be underestimated, as cellular processes are elongated and the terminal portions may be difficult to differentiate from portions of other cell types.

Vitamin A shows apple-green autofluorescence, which fades upon continued excitation. Alcian blue pH 2.5 is positive for acidic glycosaminoglycans of fat-storing cells. When present, lipid vacuoles stain positively with any of a variety of lipid stains. Semi-thin sections of Epon-embedded material stained with toluidine blue reveal the lipids of fat-storing cells as aquamarine droplets. To differentiate hepatic stellate cells from others, their location, shape, and contents (or lack thereof) have been found to be useful. Differentiation of hepatic stellate cells from endothelial cells appears to be the greatest challenge. Their common origin (mesoderm) and the presence of cytoplasm, vesicles, and cytofilaments illustrate the degree of shared features of the cell types. Alkaline phosphatase, positive in endothelial but absent in fat-storing cells, may be the strongest differentiator at present for fish.

Interhepatocytic, Perisinusoidal Macrophages

Interhepatocytic, perisinusoidal macrophages appear as single cells and are often devoid of pigment. They have been reported in rainbow trout (*Oncorhynchus mykiss*) (Hampton et al., 1985; McCuskey et al., 1986) and European brown trout (*Salmo trutta fario*) (Rocha et al., 1997). Interestingly, when quantitative

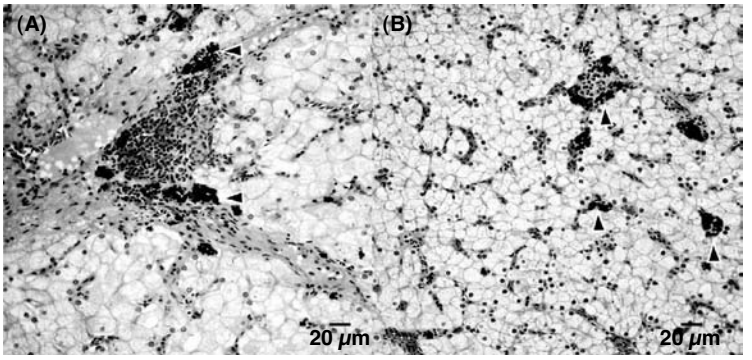

FIGURE 7.11 (See color insert following page 492.) Hematoxylin and eosin stained sections from liver of juvenile sturgeon. (A) Melano–macrophage aggregates (black arrowheads) are seen in the connective tissue tract at margins of inflammatory focus (eosinophilic granular leukocytes and mononuclear cells). (B) Liver parenchyma has smaller melano– macrophage aggregates in this juvenile. Black arrowheads point to perivascular aggregates.

assessments of livers were done in these species (Hampton et al., 1989; Rocha et al., 1997), each reported a greater number of interhepatocytic, perisinusoidal macrophages in livers of female vs. male fish.

Pigmented Macrophage Aggregates

In a recent review, the liver of mature fish was not considered to be as important to immune function as in mammals (Rice, 2001); however, macrophage aggregates are common in the liver of certain species, such as the sturgeon (*Acipenser transmontanus*) (Figure 7.11), as well as in the kidney and spleen (Agius, 1985; Kennedy-Stoskopf, 1993). The function of these cells is debated; for example, some regard these to be the phylogenetic precursors of germinal centers, but, as others have shown, they increase in number with age and also show an increase in number under oxidative stress. These aggregates are seen in the presence of disease even in younger fish (Camp, 1997; Rice, 2001). In certain species of fishes, pigments abound in these aggregates, including lipofuscin, melanin, hemosiderin, and, in some cases, ceroid (Rice, 2001). Hemosiderin is a breakdown product of hemoglobin degradation, but melanin has antioxidant properties and is a predominant feature of macrophage aggregations in channel catfish during disease states (Camp, 1997; Rice, 2001). Lipofuscin and ceroid are byproducts of saturated fat oxidation. Most authors agree that macrophage aggregates are sites of inflammation, oxygen radical formation, and lipid oxidation. In addition, the review by Rice (2001) included findings suggesting that genetic resistance to certain diseases in channel catfish were correlated with increased numbers of splenic macrophage aggregations (Camp, 1997; Camp et al., 2000).

Kupffer Cells

Some species of fish possess cells that resemble the fixed, resident macrophages of the mammalian liver (Kupffer cells). Other species, despite intense investigation, seem to lack these sinusoidal lining cells (Hampton et al., 1985; Rocha et al., 1997). Hampton et al. (1987) conducted an evaluation of resident macrophages in the livers of brown bullhead catfish (*Ictalurus nebulosus*, now *Ameiurus* sp.). These cells shared properties of the Kupffer cells of mammals, including phagocytosis and degradation of erythrocytes, as well as being strongly positive for peroxidase, intensely positive for glucose-6-phosphate dehydrogenase, and positive for phagocytosis of intravascular-induced submicron latex beads. In contrast to mammalian Kupffer cells, resident macrophages of brown bullhead showed no invaginations of the plasma membrane analogous to vermiform processes. Kupffer cells of mammals are very active in the first-order defense of the liver (Cho et al., 2000; Seki et al., 2000); in the human, they perform intricate signaling that primes other cells to react, thus providing protection against bacterial infections and hematogenous tumor metastases (Seki et al., 2000). We do not know which cells provide this or similar roles in fish. For those species examined, Kupffer cells are often absent, and much more information is needed regarding this aspect of defensive roles for teleosts livers.

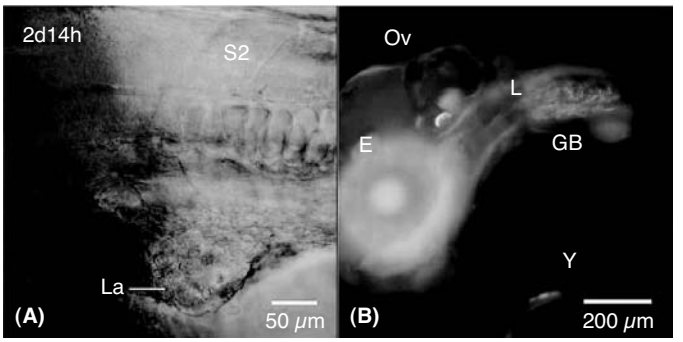

FIGURE 7.12 (See color insert following page 492.) (A) *In ovo* imaging of medaka liver anlage (La) emerging from the ventral endoderm 62 hours after fertilization. In medaka, this occurs below the first to third somite (S2). Distinct at this stage of development are hepatic tubule formations, elucidated here with the cytochrome P450-3A substrate 7-benzyloxyresorufin (7-BR). Embryonic medaka exposed to aqueous concentrations of 7-BR exhibit CYP3A activity, indicated by the red fluorescence of resorufin (the metabolic byproduct of CYP3A, via dealkylation of 7-BR) in the tubule lumens of the developing liver (red punctuate features). (B) By 5 days after fertilization, the liver (L) of medaka is found in a left lateral orientation, with the gallbladder (GB) at the liver caudal margin. Red fluorescence in the liver and gallbladder is the fluorescent CYP3A byproduct resorufin. Here, the fluorophore is seen in transit through the intrahepatic biliary passageways of the liver, with concentration in the gallbladder. E, eye; Ov, otic vesicle; Y, yolk (sac).

Selected Landmarks of Fish Liver Development

The development of fishes is considered a simple model for understanding this process in other vertebrates. Simplicity is derived from the fact that the chorion is transparent in certain fishes and provides detail for imaging key events and relationships of development *in vivo*. One species that has been used extensively as a model for studying vertebrate ontogeny is the zebrafish (*Danio rerio*), particularly for the study of liver development (Field et al., 2003; Ober et al., 2003). Another model teleost species, medaka, is an excellent experimental animal (Yamamoto, 1975). The see-through medaka is genetically deficient in pigments (Wakamatsu et al., 2001) and is being studied as a model for morphological and molecular investigations on organogenesis in the later stages of fish development and throughout the life span. Recent published and unpublished observations of the see-through medaka, made in the Hinton laboratory (Hinton et al., 2004), are used to illustrate the following: liver appearance in the embryo, anatomical position of the embryonic and larval liver, and assumptions regarding the adult vascular supply and drainage, as well as biliary function.

The liver of fishes arises from the ventral foregut. In the medaka (Figure 7.12), a bulge is seen at somites one to three on the left side of the embryo during the second day after fertilization (Iwamatsu, 2004; Iwamatsu et al., 2003) (Table 7.4). This bulge continues to grow until the left lateral longitudinal liver leaflet (L5) is formed (Figure 7.12 B). As described below, L5 is the liver of the embryo and early larval stages. The caudal-most extent of this leaflet is closely associated with a prominent, round gallbladder (Figure 7.12) the fluid of which becomes pigmented (Hinton et al., 2004), suggesting that metabolism of yolk is linked to formation of bile in the embryonic medaka. L5 is the liver during mid- and late stage embryos and extends into the first week following hatching (Hinton et al., 2004). Unpublished electron microscopic observations from this laboratory reveal that bile canaliculi are present on lateral plasma membranes of hepatocytes. In addition, *in vivo* microscopic analysis clearly shows that the L5 liver contains hepatic tubules. As is shown in Figure 7.13, transverse sections of embryonic tubules are comprised of six to eight hepatocytes whose apical plasma membranes are in contact with the tubule lumen while their basal plasma membranes face the microvasculature; therefore, even during early development, hepatocytes are polarized. Bile of the gallbladder and intestinal lumen fluoresces under ultraviolet light and may be detected through the body wall and chorion of the embryo using brightfield and widefield fluorescence microscopy (Figure 7.14). In addition, when embryos are exposed to 7-benzyloxyresorufin, a cytochrome P450 substrate, the phase I enzyme cleaves the alkyl group from

TABLE 7.4

Hallmarks of Hepatobiliary Development in ST II Medaka Compared to Related Aspects of Zebrafish Development

Dpf	Hr	Som	Hpf	Feature	Description	Medaka	Zebrafish
—	—	—	6	—	Blastula	—	HNF6[c] (6 hpf)
—	—	—	8	—	Blastula	—	HNF1β[i]
—	—	—	10	—	Blastula/gastrula	—	—
—	—	—	21	—	Gastrula	—	Ceruloplasmin[f] (16 hpf)
1	1	—	25	Neural/brain[a]	Early neurula: rudimentary brain, beak-like mass	—	Heartbeat, prox1/hhex,[d,g] FoxA2, FoxA3[e,h]
	2	—	—	Optic buds[a]	Late neurula: brain and nerve cord, Kupffer's vesicles	—	Liver bud (24–28 hpf), prox1/hhex,[d,g] FoxA2, FoxA3[e,h]
	8	4	—	Otic vesicle[a]	Otic vesicles appear; three parts of brain discernable; optic cups appear	—	Selenoprotein (28 hpf), prox1/hhex,[d,g] FoxA2, FoxA3[e,h]
	10	6	34	Optic cups[a]	Optic cups form; lenses apparent; neural fold distinct; otic vesicles distinct (no otoliths)	—	prox1/hhex,[d,g] FoxA3,[e,h] HNF4[e]
	14	9	38	Heart[a]	Heart anlage appears; hatching glands appear as cell mass	—	prox1/hhex,[d,g] FoxA3,[e,h] HNF4[e]
	20	16	44	Heartbeat[a]	33–64/min pulse; gut tube ventral; vitello-caudal vein appears on yolk sphere; blood island apparent at 6–11 somites.	—	prox1/hhex,[d,g] FoxA3,[e,h] HNF4[e]
2	2	18	50	Liver anlage	Small cell mass bulging from foregut of ventral endoderm is apparent beneath the first and third somite; blood circulation begins out of the blood island (7–15th somites); otoliths apparent; Kupffer's vesicles disappear	gata6,[b] foxA3[b]	HNF1α,[e] prox1/hhex,[d,g] HNF4,[e] HNF6,[c] Sox17[e] (53 hpf)
	10	24	58	Gallbladder	Gallbladder appears as a spherical body emerging at the caudal liver margin beneath the third somite	gata6,[b] foxA3[b]	—
	—	—	—	Gut tube	Gut tube spans 1–13 somites; caudal vein at 10–16 somites	gata6,[b] foxA3[b]	—
	—	28	62	CYP3A activity	7-Benzyloxyresorufin (BROD) activity first observed, indicative of cytochrome P4503A activity; CYP3A constitutively expressed	gata6,[b] foxA3[b]	—
	—	28	62	Hepatic tubules	Primordial tubules evident at 62 hpf (~28 somite stage), only some 12 hr after the appearance of the liver anlage at 50 hpf; sole arterial blood supply observed to feed liver from left dorsal aorta	gata6,[b] foxA3[b]	—
	16	30	64	Pancreas	Pancreas at ventral right side, slightly anterior to third somite	gata6,[b] foxA3[b]	—
	—	—	68	CYP1A activity	7-Ethoxyresorufin (EROD) activity first observed, indicative of cytochrome P4501A activity; CYP1A not constitutively expressed, induced with β-naphthoflavone and TCDD	gata6,[b] foxA3[b]	—

3	2	34	74	Pineal gland	Pineal anlage disc shaped on dorsal surface of third ventricle; sinus venosus, atrium, ventricle, bulbus arteriosus are differentiated; swim bladder emerges at third somite	gata6,[b] foxA3[b]	—
	10	35	86	Hepatic vein	At ~86 hpf, a sole prehepatic vein (PHV), the primordia of which can be observed to form in the outer yolk epithelium at 24 hpf, begins to drain the caudal liver into the left duct of Cuvier	gata6,[b] foxA3[b]	—
	10	35	86	L5 phenotype	Left lateral longitudinal liver leaflet (L5) is well distinguished at 86 hpf	gata6,[b] foxA3[b]	—
	—	—	88	Bile synthesis	Bile autofluorescence in gallbladder first observed at 88 hpf; onset of bile synthesis suspected to be earlier	gata6,[b] foxA3[b]	—
4	5	—	—	Somites complete	Somite formation completed; air bladder vacuolar body at third somites; kidneys bilateral at first somites	foxA3[b]	—
5	12	—	—	Spinal chord	Tubular spinal chord apparent	foxA3[b]	—
7	—	—	—	Gut/pericardium	Gut tube and pericardium distinguished; pericardial cavity forms; teeth are visible; linear gut tube has narrow lumen; pectoral fin movement (slow)	—	—
				Spleen	Spleen apparent at third and fourth somites	—	—
8	—	—	—	Hatching	L5 phenotype retained through 10 dpf; onset of Phase I metamorphosis; LOA, 3.3 mm	—	—
				Peristalsis	Onset of peristalsis observed; very low frequency contractions	—	—
				Phase I metamorphosis	L5 liver/gallbladder begin to descend from the upper left lateral position to ventral surface of abdominal cavity as yolk (SAC) is resorbed	—	—
				Pectoral fin activity/ immotility	Pectoral fins beat at a high rate (180–360 bpm); larvae are immotile but demonstrate a startle response	—	—
9	—	—	—	Alimentary canal	Alimentary canal opens; stomatodeal and proctodeal membranes vanish by the end of day 9; gallbladder progressively enlarges through phase I while yolk is resorbed; LOA, 4.2 mm	—	—
	—	—	—	Respiration	Onset of respiration; opercular movement suggests onset of respiration	—	—
10	—	—	—	Phase II metamorphosis	End of phase I; L5 liver/gallbladder complete descent and are at ventral surface of abdomen in L5 position; yolk resorption nearly complete; sole hepatic vein drains liver directly into sinus venosus; onset of phase II; LOA, 4.5 mm.	—	—
				Phase II metamorphosis	Onset of feeding; L5 liver/gallbladder rotate (are rotating 10–16 dpf) 90° clockwise (ventral view) to transverse position in rostral abdomen; marked vascular reorganization of viscera observed in tandem with liver/gallbladder translocation	—	—

TABLE 7.4 (cont.)

Hallmarks of Hepatobiliary Development in ST II Medaka Compared to Related Aspects of Zebrafish Development

Dpf	Hr	Som	Hpf	Feature	Description	Medaka	Zebrafish
12	—	—	—	Phase II metamorphosis	Liver and gallbladder at 45° angle, ventral view; contraction of ducts of Cuvier; merging of hepatic vein and sinus venosus	—	—
13	—	—	—	Phase II hepatic portal vein	Hepatic portal vein first observed draining the transverse gut tube mid-sagittal to the liver hilus	—	—
15	—	—	—	Phase II metamorphosis	LOA, 4.8 mm	—	—
16	—	—	—	Phase II metamorphosis completed	Liver and gallbladder achieve adult phenotype, with liver transverse in the rostral abdomen and gallbladder right lateral at liver caudal margin; typically achieved by 16 dpf	—	—
19		—	—		LOA, 4.9 mm	—	—
21	—	—	—	Adult phenotype	Adult phenotype achieved; LOA, 5.4 mm	—	—

[a] Data from Iwamatsu, T., *Mech. Dev.*, 121, 605, 2004.
[b] Data from Watanabe, T. et al., *Mech. Dev.*, 121, 791, 2004.
[c] Data from Matthews, R.P. et al., *Dev. Biol.*, in press.
[d] Data from Wallace, K.N. et al., *Genesis*, 30, 141, 2001.
[e] Data from Field, H.A. et al., *Dev. Biol.*, 253, 279, 2003.
[f] Data from Korzh, S. et al., *Mech. Dev.*, 103, 137, 2001.
[g] Data from Ober, E.A. et al., *Mech. Dev.*, 120, 5, 2003.
[h] Data from Odenthal, J., *Dev. Genes Evol.*, 208, 245, 1998.
[i] Data from Her, G.M. et al., *FEBS Lett.*, 538, 125, 2003.
[j] Data from Allende, M.L. et al., *Genes Dev.*, 10, 3141, 1996.

Abbreviations: Dpf, days post fertilization; Hr, hour; Som, number of somites produced; Hpf, hours post fertilization; LOA, loss of attachment.

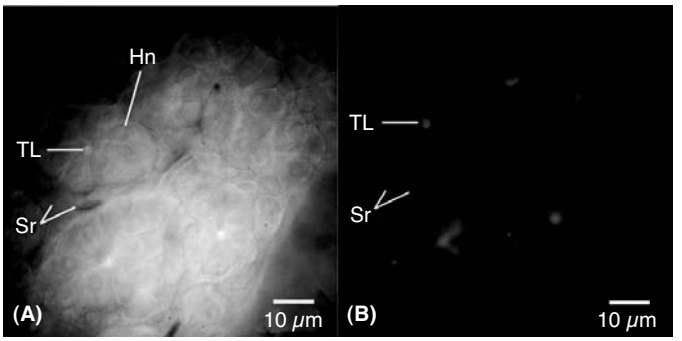

FIGURE 7.13 (See color insert following page 492.) *In vivo* microscopy of dechorionated medaka embryos 5 days after fertilization; tubular architecture in developing liver of medaka. (A) Autofluorescence of hepatic parenchyma (widefield fluorescence microscopy). Six to eight hepatocytes can be seen to, in transverse section, form the hepatic tubule. The apical membranes of hepatocytes form the tubule lumen, a central biliary passageway. Hn, hepatocyte nucleus; Sr, sinusoid with red blood cells; TL, tubule lumen. (B) Same image as A but showing TRITC fluorescence of 7-benzyloxyresofufin (7-BR) (red). 7-BR-exposed medaka embryo shows fluorescence of resorufin in lumens of hepatic tubules and in canaliculi, thus providing *in vivo* evidence for CYP3A metabolic activity and concentrative transport of fluorophore from the sinusoid to the tubule lumen.

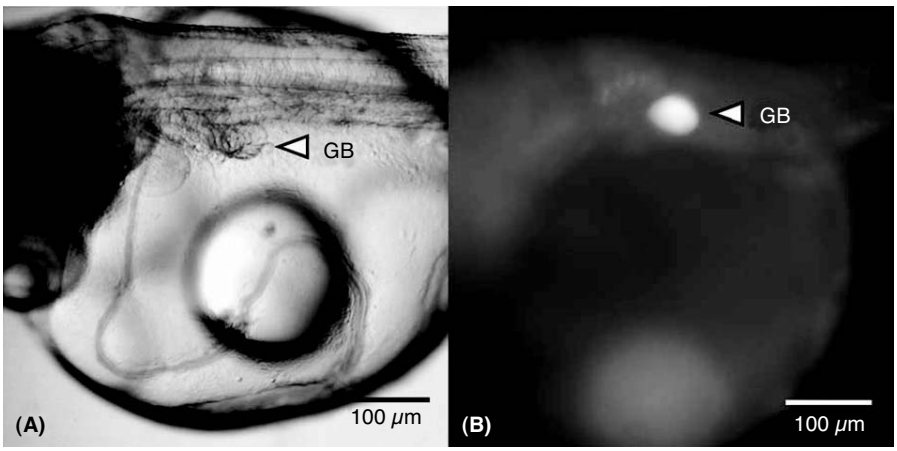

FIGURE 7.14 (See color insert following page 492.) *In vivo* microscopy (brightfield illumination) of dechorionated medaka embryo. (A) Yellow/green pigment in the gallbladder (GB) is seen. This signifies bile synthesis and export. The liver is in left upper abdominal cavity immediately dorsal to lipid droplet. The circuitous left duct of Cuvier is shown, and a portion of the media yolk vein is at the caudal margin of the yolk sac. (B) Same orientation as in A, demonstrating autofluorescence in the gallbladder and to a lesser extent in the intrahepatic passageways.

resorufin, resulting in resorufin fluorescence. Resorufin can then be detected in the canalicular network, tubule lumens, larger bile passageways of liver, gallbladder, common bile duct, and intestinal lumen (Figure 7.14). Iwamatsu et al. (2003) reported that the medaka anal aperture is not open until the fish reaches approximately 5.3 mm total length (stage 40, after hatching). This may explain the retained fluorescence of the intestinal lumen late in embryogenesis.

Another important feature of L5 is its blood supply, microvasculature, and venous drainage. *In vivo* observations (Hinton et al., 2004) have shown that a single vein (hepatic vein) drains L5, with blood flowing from the liver to the left duct of Cuvier. There is no afferent venous supply to L5; the apparent sole source of hepatic blood supply, at this time, is arterial from the dorsal aorta (Hinton et al., unpublished observations). We have observed erythrocytes tumbling through the hepatic microvasculature and passing through the hepatic vein to the left duct of Cuvier.

During the second week of larval life, an apparent metamorphosis occurs when the yolk sac is apparently completely absorbed, the gut elongates (Iwamatsu et al., 2003), and the liver (L5) assumes

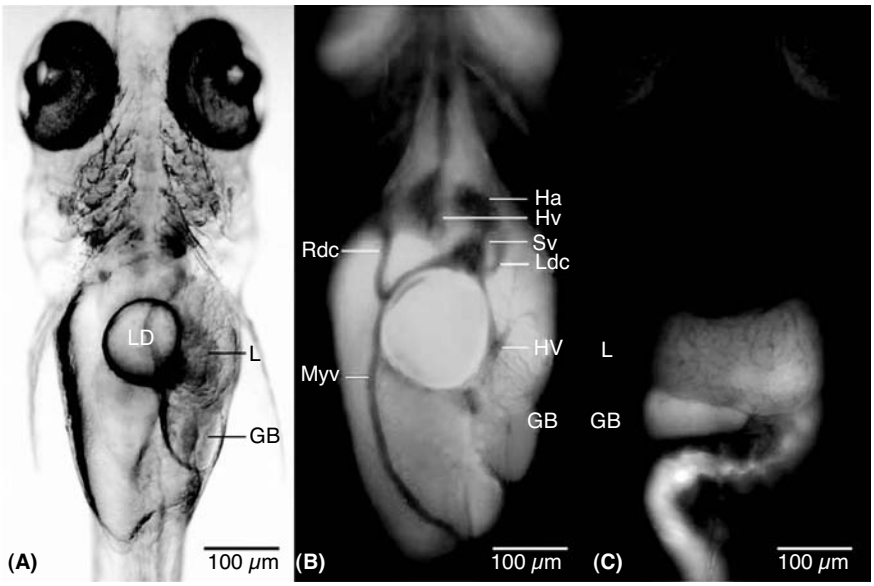

FIGURE 7.15 (See color insert following page 492.) A and B are ventral view of medaka at 10 days after fertilization. (A) Note right half of abdominal cavity is largely filled by yolk sac showing evidence of utilization of yolk. (B) DAPI/TRITC composite showing vasculature at this developmental stage. Note that the large rounded lipid droplet is maintained despite loss of most of the yolk sac. (C) Medaka at 20 days after fertilization revealing adult phenotype. Note depletion of the lipid droplet (absence). The liver is in the ventral rostral portion of abdominal cavity and partially covers the gallbladder. Autofluorescence in the gut is largely due to algae. The liver shows an extensive vascular network (dark nonfluorescent sinuous lines). GB, gallbladder; Ha, atrium of heart; HV, hepatic vein; Hv, ventricle of heart; L, liver; LD, lipid droplet; Ldc, left duct of Cuvier; Myv, median yolk vein; Rdc, right duct of Cuvier; Sv, sinus venosus.

the adult position of lying transversely in the rostral-most portion of the abdominal cavity (Hinton et al., 2004). Because the gallbladder was originally closely associated with the most caudal portion of L5 and becomes closely associated with the right side of the liver in the adult position, the liver appears to make a 90° rotation, with the original rostral portion of L5 (Figure 7.15A) becoming the left portion of the adult liver and the caudal portion of L5 becoming the right portion of the transversely positioned larval liver (i.e., stages 42 to 44) (Iwamatsu et al., 2003) and the adult liver. Afferent venous supply (the hepatic portal vein) is apparently established (Figure 7.15B) when the larval medaka liver assumes the transverse position, bringing it into close contact with the rostral portion of the vitellocaudal or median yolk vein (Hinton et al., 2004).

Specifics of Liver Function

The liver of fishes has a central function in the maintenance of organism homeostasis in that this organ regulates blood composition through: (1) synthesis, interconversion, and redistribution or removal of nutrients, vitamins, essential metals, and endogenous compounds, including hormones and blood proteins; and (2) removal of endotoxins, particulates, or metabolic wastes, such as bilirubin and ammonia. For example, after meals, in the postprandial absorptive stage, the liver takes up nutrients from the plasma. Later, stored nutrients are released during catabolic states such as starvation or stress. In this way, the liver smoothes out potentially large fluctuations in blood composition and allows nutrients to reach peripheral tissues. The liver further synthesizes and secretes a number of proteins such as fibrinogen and albumin; also, in female trout and medaka, the egg-yolk protein vitellogenin and eggshell membrane protein choriogenin are produced. In the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*), however, the eggshell membrane proteins (choriogenin, zona radiata protein, zona pellucida, vitelline envelope proteins) are produced in the maturing oocytes (Arukwe and Goksøyr, 2003; Carvan et al., 2007).

The important metabolic role of the liver is related to its unique location within the systemic circulation. This location is also responsible for the importance of the liver in the uptake, storage, metabolism, redistribution, and excretion of environmental toxicants and makes it at the same time a target of toxic substances. The livers of fishes can store large amounts of either glycogen or lipid. The type of storage product may change with species. Some species, such as salmonids, preferably store glycogen in the hepatocytes; others, such as cod, deposit large amounts of lipids, and cyprinids can shift between the deposition of glycogen or lipid (Bohm et al., 1994; Fujita 1985; Welsch and Storch, 1973). Also, physiological factors such as sex (Braunbeck, 1998; Peute et al., 1978) or environmental factors such as nutrition or temperature can strongly change the quantity and type (glycogen/lipid) of hepatic energy stores (see, for example, Braunbeck et al., 1987; Berlin and Dean, 1967; Gas and Serfaty, 1972; Segner and Braunbeck, 1988, 1990a; Segner and Moller, 1984; Segner and Witt, 1990). Such changes are indicative of an in-depth reorientation of hepatic metabolism in response to endogenous or exogenous change, and the changing metabolic state can directly or indirectly influence the toxicant sensitivity of the fish (Braunbeck and Segner, 1992; Braunbeck et al., 1989; Koehler 2004).

Liver Metabolism

Aspects of liver intermediary metabolism, liver xenobiotic metabolism, and experimental *in vitro* hepatic systems have been covered in detail by other reviews and in other chapters of this book. Here, we summarize a few issues that are important for our coverage in this chapter. We refer interested readers to the following relevant reviews: For *in vitro* metabolism, see Mommsen et al. (1994), Pesonen and Andersson (1997), Denizeau (1998), Monod et al. (1998), and Segner (1998). For information on aspects of *in vivo* liver xenobiotic metabolism, see Andersson and Forlin (1992), Stegeman et al. (2004), and Schlenk et al. (2006). For intermediary metabolism and aspects of liver metabolism, see Cowey and Walton (1982), Moon and Foster (1995), Navarro and Gutierrez (1995), Hemre et al. (2002), and Moon (2004).

Research over the past two decades has established that the livers of fish contain many of the same metabolic pathways and enzymes known for mammalian liver (Cowey and Walton, 1982; Moon et al., 1995); however, important differences exist (for more details, see Hinton et al., 2001). An important difference between fish and mammalian liver with respect to toxicant action is the apparent absence of a functional metabolic zonation. In the mammalian liver, storage products such as glycogen and lipid as well as many metabolic enzymes are heterotopically distributed in the parenchyma. The distribution patterns appear to be related to the direction of microvascularization. The capacity for oxidative metabolism (e.g., β-oxidation of fatty acids, amino acid catabolism, or oxidative energy metabolism) is higher in the periportal than in the perivenous zone or centrolobular (zone 3 of the liver acinus model in mammals) (Jungermann and Katz, 1989). Accordingly, the upstream cells show greater mitochondria and larger cristae area than do the downstream cells (Loud, 1968). It is supposed that the concentration gradients of oxygen, hormones, and substrates in the sinsusoidal bloodstream lead to the expression of different enzyme levels in the bordering hepatocytes. The concept of metabolic zonation (Jungermann and Katz, 1989; Teutsch, 1981) suggests that the biological significance of this functional heterogeneity might be to enable the liver to perform antagonistic metabolic functions at the same time.

The distribution of enzymes and storage products within the liver parenchyma of fish has been determined by enzyme histochemistry in frozen sections and by digitonin pulse infusion. For most enzymes studied, histochemical investigations were not able to demonstrate a heterotopic distribution in the liver parenchyma of fish (Burkhardt-Holm et al., 1993; Hampton et al., 1985; Schar et al., 1985; Segner and Braunbeck, 1988) (Table 7.5). Glycogen phosphorylase is the only enzyme for which all studies report a heterogeneous distribution in fish liver; however, it is not clear to what extent the observed phosphorylase pattern is determined by, or associated with, the hepatic microvasculature.

To separate hepatocytes from sites adjacent the afferent vasculature from those adjacent the efferent vasculature, a short pulse of cytotoxic digitonin from either the portal (anterograde) or hepatic vein (retrograde) is used. Subsequently, cell isolates presumably enriched in periportal or perivenous liver cells may be prepared and analyzed for their metabolic properties. For teleosts, such studies have been done with rainbow trout (*Oncorhynchus mykiss*) (Mommsen et al., 1991), toadfish (*Opsanus tau*) (Mommsen and Walsh, 1991), and catfish (*Ictalurus melas*) (Ottolenghi et al., 1991). None of the cited studies observed

TABLE 7.5

Heterogeneous Distribution of Metabolic Enzymes in Liver Parenchyma

Enzyme	Rat[a]	Rainbow Trout[b]	Golden Ide[c]	Carp[d]
Glucose-6-phosphatase	Zonation	Partly	No zonation	No zonation
Glycogen synthase	Zonation	Not determined	No zonation	Not determined
Glycogen phosphorylase	Zonation	Zonation	Zonation	Zonation
Glucose-6-phosphate dehydrogenase	Zonation	No zonation	No zonation	No zonation
Malic enzyme	Zonation	Not determined	No zonation	No zonation
Lactate dehydrogenase	Zonation	No zonation	Not determined	No zonation
Succinate dehydrogenase	Zonation	No zonation	Not determined	Not determined
NADPH tetrazolium reductase	Zonation	Not determined	Not determined	No zonation

[a] Data from Schar et al. (1985) and Jungermann and Katz (1989).
[b] Data from Hampton et al. (1985).
[c] Data from Segner and Braunbeck (1988) and Burkhardt-Holm et al. (1993)
[d] Data from unpublished work of Segner.
Note: Enzyme distribution was studied by means of enzyme histochemistry.

significant differences in enzyme activities between the two cell populations; however, in rainbow trout, small differences existed with respect to the rates of gluconeogenesis (Mommsen et al., 1991). Significant differences in metabolic enzyme activities were only detected when trout liver cells were not isolated with respect to their topographical localization but with respect to cell size (Mommsen et al., 1991). This result may indicate the existence of a cell-to-cell microheterogeneity in fish liver that is different from the parenchymal zonation that exists in mammalian liver or the presence of smaller, nonhepatocytic cells.

In conclusion, the available evidence for metabolic zonation in fish liver is weak. Although liver subpopulations appear to exist to some extent, the distribution pattern clearly differs from that seen in mammals. The apparent absence of metabolic zonation in teleost liver might be explained by the irregular and largely undefined hepatic microvasculature in fish (see above).

The absence of a functional metabolic zonation in fish liver has consequences with respect to toxicant action. A zonal toxicity can result from the heterogeneous distribution of metabolic enzymes, oxygen, etc. in mammalian liver (Treinen-Moslen, 2001); for example, the toxicity of CCl_4 that is related to the metabolic production of the CCl_3 radical is mainly expressed in the perivenous zone (centrolobular region, or acinus zone 3), as hepatocytes of this zone have more P450-dependent enzymes and lower O_2 levels. In fish, functional zonation of the liver parenchyma is lacking; consequently, no zonal toxicity has been observed. This does not mean that toxicity has not occurred, because when cells are evaluated *in vitro* toxicity is verified by the leakage of cytosolic enzymes to media in culture systems (Rabergh and Lipsky, 1997).

Liver cells are important for xenobiotic metabolism and have high constitutive activities of many phase I enzymes that convert xenobiotics to reactive electrophiles, including cytochrome P450 isozymes, alcohol dehydrogenases, and quinone reductases. Also, hepatocytes have a rich collection of phase II enzymes that add a polar group to a molecule, rendering it more soluble in water and enhancing its removal from the body. In fish, the best-studied biotransformation enzyme is cytochrome P4501A, which shows the highest specific activities in the liver (Hahn and Stegeman, 1994). The enzyme is localized in hepatocytes, biliary epithelial cells, and vascular endothelial cells. In contrast to mammals, where cytochrome P4501A shows a heterogeneous distribution throughout the liver parenchyma, no zonation can be observed in teleost liver (Lorenzana et al., 1989; Ortiz-Delgado et al., 2002; Smolowitz et al., 1992). Subcellularly, cytochrome P4501A is localized at the membranes of the rough endoplasmic reticulum and the outer leaflet of the nuclear envelope (Lester et al., 1993).

Phase I Metabolism: CYP1A

Hepatic CYP1A expression of fish can be induced by exposure to specific environmental contaminants, including halogenated aromatic hydrocarbons (e.g., dioxins, furans, polychlorinated biphenyls) and polyaromatic hydrocarbons (PAHs). The induction response is mediated via an intracellular receptor, the dioxin or aryl hydrocarbon receptor (AhR; see below). Due to the specific ligand–receptor interaction required for

activation of the AhR pathway and AhR-dependent CYP1A induction, this biochemical response has been used extensively as a biomarker of exposure of fish to dioxin-like contaminants (Hahn, 2002; van der Oost et al., 2003; Whyte et al., 2000) and has been used in a range of field biomonitoring studies (Behrens and Segner, 2005; Collier et al., 1995; Goksøyr and Förlin, 1992; Payne and Penrose, 1975). Typically, CYP1A induction, measured as catalytic 7-ethoxyresorufin-*O*-deethylase (EROD) activity, as CYP1A protein, or as CYP1A mRNA, is assessed in the liver because specific CYP1A expression is highest in this organ.

Although CYP1A is induced in various resident liver cell types, responding cells appear to differ in their induction response to xenobiotics. Environmental exposure led to a particularly strong increase of cytochrome P4501A immunoreactivity in the biliary and endothelial cells of cod and flounder, while hepatocyte staining remained weak (Husoy et al., 1996). In contrast, in lemon sole (*Microstomus kitt*), the cytochrome P450 induction response was stronger in hepatocytes than in vascular cells (Husoy et al., 1996). In scup, 3,3′,4,4′-tetrachlorobiphenyl treatment strongly increased CYP1A immunoreactivity in both hepatocytes and nonhepatocytes (Singh et al., 1996). It is possible that the cell-specific induction response varies with the type of inducer (readily vs. slowly metabolized compounds), dose of inducer, or fish species (Anulacion et al., 1998; Goksøyr and Husoy 1998; Ortiz-Delgado et al., 2005). Another relevant factor influencing hepatic CYP1A response could be the exposure route. A stronger increase of CYP1A immunostaining occurred in hepatocytes of mummichog (*Fundulus heteroclitus*) when exposure took place via the water than by the dietary route (Van Veld et al., 1997).

Hepatic CYP1A induction is a measure of the exposure of fish to dioxin-like chemicals; it is not a direct measure of the ability of a species to metabolize these compounds. A direct correlation was demonstrated in mammals and birds between ability to metabolize 3,3′,4,4′-tetrachlorobiphenyl and their hepatic EROD activity; however, no such relation was seen in rainbow trout and flounder (Murk et al., 1994). This finding possibly reflects species differences in the participation of different CYP isoenzymes in the metabolism of the toxicant. In line with this speculation is the observation that the liver of turbot exposed to benzo(*a*)pyrene produced different metabolites before and after induction of hepatic EROD activity (Telli-Karakoc et al., 2002).

Phase I Enzymes Other than CYP1A

Phase I metabolic enzymes have not been as intensively studied in fish liver, but recent advances are noteworthy. With the completion of genome sequences for several teleosts, including medaka, zebrafish, pufferfish, and stickleback, full complements of CYP gene families are being discovered (Nelson, 2003; http://drnelson.utmem.edu/CytochromeP450.html). Although the functionality for many of these enzymes remains to be determined, several have been investigated using recombinant systems such as CYP3A38/40 (Kashiwada et al., 2005) , CYP2N (Oleksiak et al., 2000), CYP2K1 (Yang et al., 2000), CYP2M1 (Yang et al., 1998), and CYP3A27 and CYP3A45 (Lee and Buhler, 2002, 2003). In general, the catalytic activities and substrate specificity of teleost CYPs are highly similar to their mammalian homologs. Examples include the preference of CYP1A for aromatic hydrocarbons, CYP3A for steroidal compounds, and CYP2 for arachidonic acid, lauric acid, and sex steroid hormones. In some instances, however, activities may be specific to teleosts. Mammalian forms of CYP3A preferentially catalyze 6β-hydroxylation of testosterone with lower levels of the 2β-, 15β-, and 1β- hydroxides (Krauser et al., 2004). In comparison, teleost CYP3A forms catalyze testosterone hydroxylation at the 6β-, 2β-, and 16β- positions (Kullman and Hinton, 2001; Lee and Buhler, 2002, 2003), illustrating an inherent difference in catalytic profiles that exists between teleost and mammalian species. Any physiological significance of this difference has yet to be determined. Often, the optimal requirements for cofactors, including CYPOR, cytochrome b_5, lipids, and temperature, differ among teleosts. This is not surprising given the fact that these organisms inhabit a range of environmental conditions from extreme cold to tropical temperatures and fresh to seawater (Kashiwada et al., 2005). The availability of modern molecular techniques has made it much easier to identify specific isoforms and has thus stimulated research on the CYP isoenzymes in fish. With these new technical developments, a more in-depth characterization of the phase I metabolism of xenobiotics in fish liver will become possible. Of great interest is a further understanding of CYP temporal and spatial expression, the mechanisms of gene regulation, and catalytic function, much of which remains unknown for many of these enzymes.

Hepatic Phase II Metabolism

During phase II reactions, xenobiotic metabolites produced from the first phase are conjugated to polar endogenous substrates (e.g., sulfate, glucuronide), facilitating their subsequent elimination from the body via bile or urine (Stegeman, 1989). Biliary excretion is often chemical specific (see detailed coverage below). Low-molecular-weight compounds are poorly excreted into bile, but high-molecular-weight xenobiotic compounds conjugated with sulfate and glucuronide are readily excreted into the bile (Klaassen and Watkins, 1984). Several types of petroleum hydrocarbon metabolites have specific fluorescence properties, and exposure of fish (e.g., Atlantic cod, Atlantic salmon, English sole) to crude oil results in increases in bile fluorescent aromatic compounds (FACs) (Aas et al., 2000; Gagnon and Holdway 2002; Krahn et al., 1986). Significant correlation has been established between the prevalence of hepatic lesions and mean biliary metabolite concentration in fish, and analysis of FAC in fish bile is recommended as a sensitive method for detection of PAH contamination from both pyrolytic and petroleum origin (Krahn et al., 1986).

In general, conjugation reactions, such as glucuronidation or glutathione conjugation, have not received sufficient attention, and further studies must be conducted in the future. Just as was shown for phase I metabolism, the liver is quantitatively the most important site for phase II metabolism in fish (Clarke et al., 1991). In mammals, phase II enzymes such as glutathione *S*-transferases and UDP-glucuronyltransferases represent multigene families, and there is evidence that this applies for fish as well, although the characterization of the various isoenzymes requires more attention (Leaver et al., 1992, 1993). In field studies of fish from contaminated sites, phase II conjugates of xenobiotics were detected in bile. Examples include fuel-oil PAHs (Collier and Varanasi, 1991), phthalate ester plasticizers (Melancon and Lech, 1983), and the pulp mill constituents chlorophenols and resin acids (Hardig et al., 1988; Oikari et al., 1985; Stuthridge et al., 1997). The importance of phase II metabolism for the bioconcentration and toxicity of xenobiotics in fish may be exemplified by an organophosphorous compound. In several fish species, dimethylphosphorothioates are rapidly metabolized in the liver and eliminated via bile as glutathione conjugates. In addition, this rapid phase II metabolism is associated with lower bioconcentration and lower toxicity than observed for related organophosphorous compounds that are metabolized by the oxidative system (Debruijn et al., 1993). Another example is provided by the lampricide 3-trifluoromethyl-4-nitrophenol. The selective toxicity of this substance to sea lampreys was found to be related to a greatly reduced capacity of this species to glucuronidate the compound (Lech and Statham, 1975).

To study liver xenobiotic metabolism, *in vitro* preparations of fish liver cells have proven to be a valuable experimental model. Isolated hepatocytes can be kept as fresh isolates (Moon et al., 1985), as longer term monolayers (Pesonen and Andersson, 1997; Segner 1998), or as aggregate cultures (Cravedi et al., 1996). These preparations have the advantage of making it possible to analyze the fate of xenobiotics specifically in the liver, in the absence of systemic influences. Further, they offer the possibility for interspecies comparison of chemical metabolic conversion under fairly comparable conditions (Coulombe et al., 1984; Cravedi and Baradat, 1991; Murk et al., 1994). Fish hepatocytes *in vitro* are valuable tools for study of induction of biotransformation enzymes as well as the formation of xenobiotic metabolites (as reviewed in Segner and Cravedi, 2001). Generally, the metabolite pattern produced by fish liver cells *in vitro* agrees well with the *in vivo* metabolism of the substances, although possible differences should not be overlooked when extrapolating from *in vitro* to *in vivo* hepatic metabolism (Cravedi et al., 1999; Morrison et al., 1985; Nishimoto et al., 1992). An interesting example of such an *in vitro* and *in vivo* differences was provided by Cravedi et al. (2001), who found the toxicologically relevant hydroxylamine metabolite of 2,4-dichloroaniline in the *in vitro* hepatocyte preparations but not *in vivo* in the bile fluid. The likely explanation for this difference is that the hydroxylamine metabolite is unstable; therefore, it can be detected only in the culture media, which are processed after a 1-hour incubation period, while the bile is sampled only at 24-hour intervals.

Nuclear Receptors in Liver Biology

Major functions of livers—the uptake, metabolism, storage, and redistribution of nutrients and endogenous molecules; metabolism of xenobiotics; and formation and excretion of bile—are regulated by nuclear receptors (NRs). Mammalian liver NRs are endogenous sensors, regulating many of the above

hepatic functions. With certain large gaps in information, a qualitatively similar story is unfolding in fish. Importantly, livers of oviparous species have an additional hepatic function as an integral component of the reproductive axis. In response to estrogen, hepatocytes in these organisms synthesize vitellogenin, a yolk precursor protein, and choriogenin, a component of the outer egg envelope. Thus, hepatic NRs of fishes will likely perform key roles, as above, but we need to know more about the molecular mechanisms of NR action, their roles in critical liver functions, and the molecular mechanisms associated with gene regulation.

Mammalian Liver Nuclear Receptors

Nuclear receptors are ligand-dependent transcription factors that bind lipophilic signaling molecules and result in the control and expression of target genes. Mammalian NRs facilitate cellular responses to endogenous and exogenous ligands by coordinating complex transcriptional responses (Mangelsdorf and Evans, 1995). The NR superfamily includes receptors for multiple endobiotics, such as steroid hormones, retinoids, thyroid hormone, vitamin D, bile acids, and prostaglandins. Also of importance are exogenous ligands, including dietary components and xenobiotics.

Nuclear receptors commonly share a conserved *N*-terminal DNA-binding domain (DBD) and a *C*-terminal ligand-binding domain (LBD). The DBD contains two zinc-finger motifs that form a single structural domain containing an α-helix reading head that controls specific DNA sequence recognition (Lin and White, 2004). The LBD confers ligand specificity and also contains a ligand-inducible transactivation function (AF2) essential for transcriptional activation. In the absence of a ligand, NRs are associated with a nuclear receptor corepressor complex, resulting in inhibition of the basal transcription activity of the associated promoter (Mangelsdorf and Evans, 1995; Ordentlich et al., 2001). Corepressor proteins (SMART, NCoR) couple non-liganded, DNA-bound NR to enzymes with histone deacetylase activity, resulting in chromatin condensation and a subsequent repression of gene expression (Polly et al., 2000). Ligand binding causes a conformational change within the carboxy-terminal LBD (Rochel et al., 2000), resulting in the release of corepressors and facilitating protein–protein interactions with dimerization partners, coactivators, and mediator proteins (MEDs) (Glass and Rosenfeld, 2000). Coactivators couple ligand-activated NRs to enzymes displaying histone acetyltransferase activity, thereby facilitating chromatin remodeling. In subsequent steps, ligand-activated NRs interact with additional MED family members to form a bridge to the RNA polymerase II (Pol II), resulting in transcription activation. NRs bind to DNA as homodimers or heterodimers (usually with RXR) to hexameric response elements (HREs) (5′-AG(G/T)TCA-3′), arranged as direct repeats (DRx), inverted repeats (IRx), or everted repeats (ERx) separated by "x" base pairs.

The NR superfamily in mammals is composed of approximately 50 functional genes, 48 in humans, 47 in rats, and 49 in mice (Zhang et al., 2004). Interestingly, teleost fishes have a larger complement of NR genes (68 in pufferfish) due to whole genome duplication events (Maglich et al., 2003). The current official nomenclature for NRs divides the superfamily into seven families (NR0–6), with further subclassification designated by alphanumeric characters representing sequence similarities (Bertrand et al., 2004).

Fish Nuclear Receptors with an Emphasis on Teleosts

Genomics efforts in pufferfish, zebrafish, and medaka have revealed orthologs for most mammalian NRs, including those for steroid hormones, orphan receptors, and members of all seven NR families. In fact, most NRs from teleosts, some cartilaginous fishes, and other lower vertebrates exhibit strong homologies to mammalian sequences; however, certain structural modifications suggest functional differences that may reflect adaptations arising with the movement of vertebrates from aquatic to terrestrial environments. In addition, genome duplication resulted in a greater number of NRs in teleosts than in most mammals (Venkatesh, 2003; Volff, 2005); for example, those NRs for which duplicate genes exist include the vitamin D receptor (VDR), farnesol X receptor (FXR), glucocorticoid receptor (GR), and estrogen receptor (ER), among others (Maglich et al., 2003). As a result, questions regarding molecular function and diversification of paralogus sequences remain. Gene duplication events have different outcomes. Formation of a nonfunctional, duplicate gene may result. Subfunctionalization of duplicated genes may occur followed by degenerate mutations that modify expression or activity patterns of the single ancestral

gene. Also, neofunctionalization may occur in which duplication of one daughter gene with the ancestral function occurs while the other acquires new functions (He and Zhang, 2005). It seems likely that subfunctionalization or neofunctionalization of NR paralogs may have been involved in the generation of fish variability. Additionally, from a molecular perspective, multiplicity of nuclear receptors may be an important factor that contributes to both signal diversification and specification (Gronemeyer et al., 2004). In this capacity, gene paralogs may impart novel functions that suit the unique physiological demands of inhabiting an aquatic environment or specific reproductive strategies. As discussed below, additional differences are noted between teleost and mammalian NRs, including structural changes resulting in differential ligand-binding characteristics (peroxisome proliferator-activated receptor [PPAR] alpha and gamma), absence of particular NRs, constitutive androstane receptor (CAR), or quantitative differences in ligand binding (ER).

The NR1I Subfamily

The NR1I subfamily has received a great deal of attention due to an essential role in regulating phase I and II genes involved in xenobiotic metabolism. For many years, the mechanisms governing cytochrome P450 gene induction following xenobiotic exposure remained elusive. AhR–CYP1A interactions were well described, but the processes by which induction of CYP2, CYP3, and CYP4 families occurred had yet to be determined (Hahn et al., 2005). With the discovery of the nuclear receptors CAR and pregnane X receptor (PXR), our mechanistic understanding of CYP regulation was greatly enhanced. Both CAR and PXR are low-affinity (high substrate concentration) xenosensors in mammals, capable of regulating genes associated with the metabolism, transport, and elimination of exogenous substrates. Human and rodent PXR can be activated by a variety of compounds known to induce hepatic P450 enzymes, including prescription drugs, steroids, and bile acids, and by several suspected endocrine-disrupting compounds (Hurst and Waxman, 2004; Masuyama et al., 2002; Moore et al., 2002). This broad substrate promiscuity of PXR is due to a 50- to 60-amino-acid insert between helix 1 and helix 3. The position of this insert was confirmed by x-ray examination, which revealed an unusually large ligand-binding pocket capable of accommodating a wide range of lipophilic ligands (Moore et al., 2002). Significant species differences arose from structural changes in the LBD resulting in an array of ligand-binding and transactivation specificities. PXR was initially identified as a candidate xenobiotic receptor based on its association with the induction of the hepatic P450 enzymes CYP2B and CYP3A (Savas et al., 1999; Waxman, 1999; Xie and Evans, 2001). Subsequent work proved that PXR mediates metabolism and the elimination of harmful hepatotoxic compounds by the concerted action of the oxidative phase I CYP enzymes, phase II conjugating enzymes, and drug transporters (Willson and Kliewer, 2002). To date, PXR is involved in the regulation of transcription targets for phases I, II, and III, including CYP2B, CYP3A, UGT1A1, MDR1, CYP24, and 5-AAS (Reschly and Krasowski, 2006). The development of PXR transgenic and knockout mice, the use of microarrays, and being able to screen mammalian genomes for putative PXR response elements have helped identify numerous PXR genes and their targets, including cell growth and differentiation and heme biosynthesis, among others, thus raising the possibility of a broader physiological role for PXR (Hartley et al., 2004).

The molecular characteristics of CAR and PXR have been compared in mammals. CAR differs from PXR in ligand-binding affinities, cytoplasmic localization, basal activity, and transrepression by specific ligands, including androgen steroids (Kakizaki et al., 2003). CAR does not contain the helix 1–3 insert as PXR does, with the result that a smaller ligand-binding pocket in CAR restricts the diversity of suitable ligands. The action of CAR as a xenobiotic receptor was confirmed by several studies examining CAR-dependent gene transcription (Ueda et al., 2002). Unique to the NR1I nuclear receptor family, CAR can undergo ligand-independent activation following treatment with phenobarbital (PB). The manner in which this is achieved may involve PB initiating a phosphorylation-dependent signal cascade that leads to translocation of CAR from the cytoplasm to the nucleus without direct PB binding (Kodama and Negishi, 2006). Additionally, CAR and PXR share overlapping transcriptional targets through recognition of similar DNA response elements. This cross-talk is hypothesized to operate as a metabolic safety net between receptors (Maglich et al., 2002); however, given the striking differences in their pharmacologic profiles, these receptors may have evolved to serve distinct physiological roles (Moore et al., 2002).

Pregnane X Receptor (PXR) in Fish

While the NR1I family has been extensively studied in mammalian species, much fewer functional data for these members of the NR family are available for teleosts and for cartilaginous fishes. PXR has been identified in pufferfish, zebrafish, and medaka, and mining their genomes suggests the presence of a single PXR/CAR-like gene in each species. These teleost receptors retain similarity to mammalian PXR, having a 50- to 60-amino-acid insert between helices 1 and 3 (Moore et al., 2002), and may be a functional precursor of mammalian PXR. Interestingly, transactivation studies with a chimeric Gal4–zfLBD system from zebrafish PXR demonstrated limited diversity of functional ligands. While responsive to prototypic PXR ligands (nifedipine, phenobarbital, clotrimazole, and 5β-pregnane-3,20-dione), transactivation was low in comparison to mammalian PXR. These differences, however, may reflect the high degree of sequence variation in the ligand-binding domain. Comparative cross-species analysis of PXR suggests a high degree of variation at this site (Reschly and Krasowski, 2006). Analysis of endogenous biliary ligands demonstrated that zebrafish PXR activation was restricted to the C27 bile alcohol cyprinol sulfate, the major zebrafish bile salt. By comparison, C24 bile acids (found in birds and mammals) were ineffective (Krasowski et al., 2005a). Fish bile acids are highly variable, with some species containing C27 bile alcohols and acids and others C24 bile alcohols and acids. Some, such as the medaka, contain both C27 and C24 bile acid components (Lee Hagey, pers. commun.). The role of PXR as an endogenous sensor for bile acids may be highly species specific and subject to selective adaptations depending on the metabolic requirements.

From a toxicological perspective, it is important to know whether PXR is associated with induction of teleost P450 enzymes, specifically CYP3A. In one study, typical mammalian PXR receptor agonists, including dexamethasone (DEX), the macrocyclic antibiotic rifampicin (RIF), and the synthetic steroid pregnenolone-16α-carbonitrile (PCN), were seemingly ineffective at altering teleost CYP gene transcription (Celander et al., 1989, 1996). In tilapia (*Oreochromis niloticus*), however, PCN treatment resulted in a twofold induction of CYP3A proteins (Pathiratne and George, 1996). Similarly, exposure of zebrafish to PCN resulted in a slight increase in PXR, CYPA, and MDR1 gene transcription. No change was observed with other prototypic PXR agonists, including the antimycotic clotrimazole (CTZ) or the antianginal drug nifedipine (NIF) (Bresolin et al., 2005). 4-Nonylphenol, the major byproduct of alkylphenol ethoxylates, induces CYP3A in several fish (Hasselberg et al., 2005; Kullman et al., 2004). In a recent study, nonylphenol-induced expression of CYP3A correlated with an increased expression of salmon PXR, suggesting possible coregulation (Meucci and Arukwe, 2006). A similar finding was observed with DDE, illustrating the broad substrate affinity of Atlantic salmon PXR to environmental contaminants (Mortensen and Arukwe, 2006). In a recent review by Goksøyr (2006) on endocrine disruptors in the marine environment, the nature of the effect of such compounds was considered to be due to dose-dependent routing and cross-talk between different classes of nuclear receptors. Zebrafish CYP3A65 transcription is enhanced by administration of DEX and RIF (Tseng et al., 2005). Interestingly, CYP3A65 is additionally responsive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), indicating possible cross-talk between PXR and AhR. To date, however, there exists only indirect evidence that teleost PXR is associated with CYP expression based on similarity in response to mammalian PXR ligands. Although gene sequences for PXR have been identified, few functional data exist regarding the role of PXR in the transcriptional activation of hepatic metabolic genes and their physiological significance. *In vitro* transactivation studies and identification of cognate hormone response elements upstream of teleost CYP3A genes may provide some evidence that nuclear receptors are involved in transcriptional regulation; however, this has not been determined to date.

Constitutive Androstane Receptor (CAR) in Fish

Genomic data have revealed a wide diversity of CYP2 genes in several fish species (Nelson, 2003). Findings suggest that this CYP family has greatly diversified in fishes as compared to mammals; however, both structure and function have been conserved. To date, the functional properties of few teleost CYP2 genes have been thoroughly investigated. Heterologus expression of CYP2M1 and CYP2P demonstrated a diversified role in xenobiotic and endobiotic metabolism (Oleksiak et al., 2003; Yang et al., 1998). Unlike mammals, however, teleosts exhibit an apparent lack of CYP2B and CYP2 gene induction

following exposure to the potent mammalian CYP2B/CYP2C inducers phenobarbital and 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (Elskus and Stegeman, 1989; Kleinow, 1990). In mammals, induction of CYP2B proceeds through activation of CAR (Honkakoski and Negishi, 1997; Honkakoski et al., 1998a,b). Curiously, efforts to clone CAR from numerous teleosts have proven unsuccessful, and the mining of several teleost genomes suggests an obvious lack of CAR. In agreement with genome sequencing, experimental evidence has demonstrated that in teleost species only a single PXR/CAR-like gene is present. Iwata et al. (2002) identified a single immunoreactive protein from scup liver cytosol with CAR-like antigenic properties; however, no induction of CYP2-like protein or translocation of such protein was observed following treatment with TCPOBOB. Phylogenetic analysis suggests that NR genes appear to be subject to strong purifying selection, particularly in the DNA-binding domains (Krasowski et al., 2005b); however, estimates of the ratio of the nonsynonymous to synonymous nucleotide substitutions for the NR1I subfamily indicate that only PXR and CAR have undergone recent positive selection. Current convention suggests that CAR arose with duplication of a premammalian PXR (Krasowski et al., 2005b). It is most likely that teleosts lack a CAR-like receptor. In fishes, however, other members of the NR1I family (PXR and VDR) may demonstrate broader ligand profiles. An alternative hypothesis is that CAR has been lost in some teleosts species.

Vitamin D Receptor (VDR) in Fish

The vitamin D receptor (VDR) has been cloned from a number of teleosts, including flounder, trout, zebrafish, salmon, carp, sea bass, and medaka. Recently, VDR was also cloned from the jawless agnathan lamprey, which represents the most ancient lineage among extant vertebrates (Whitfield et al., 2003). Prior to the cloning of VDR in aquatic organisms, general convention held that the $1,25(OH)_2D_3$–VDR system originated in terrestrial animals. It is well known that vitamin D is one of the oldest hormones and is present in the earliest life forms, including phytoplankton and zooplankton (Holick, 2003a,b). Workers have questioned the significance of the capacity for vitamin D production in early eukaryotes, and one hypothesis is that vitamin D photoproducts serve as a photon sink, protecting DNA, RNA, and proteins from the damaging effects of high-energy ultraviolet radiation.

Certain fish are very rich sources of vitamin D as compared to higher vertebrates, which have insignificant amounts of this vitamin. This high level of vitamin D is found both in teleosts and in the cartilaginous elasmobranchs, signifying that high levels are not related to skeletal calcium. This finding led to speculation that function of vitamin D and the VDR in fish may be different from its known classical functions in terrestrial animals (Rao and Raghuramulu, 1999a,b). Moreover, endocrine physiology in fish has adapted to an external aquatic environment that provides a constant source of calcium ion; thus, the mechanisms regulating calcium mobilization may differ between aquatic and terrestrial organisms and between marine and freshwater fish species (Power et al., 2002). To date, however, the role of vitamin D in calcium regulation in teleosts remains equivocal. Studies in different fish species are contradictory, with a majority of evidence suggesting that VDR does not classically mediate calcium mobilization from fish enterocytes (Larsson et al., 2003; Nemere et al., 2000; Rao and Raghuramulu, 1999a). These results may reflect the enormous diversity in teleost physiology.

Recently, in mammals, VDR had been demonstrated to act as a low-affinity receptor for the secondary bile acid lithocholic acid (Makishima et al., 2002). This action may act as a hepatoprotective mechanism similar to that observed with FXR and PXR for the metabolism and export of toxic bile acids. To date, VDR exhibits receptor cross-talk with PXR for the transcriptional regulation of the broad-specificity hepatic and intestinal phase I enzymes CYP2B6, CYP2C9, and CYP3A. Thus, other than the high-affinity endocrine effects, VDRs exhibit considerable overlap in metabolic transcriptional targets. The role of teleost VDR in regulating hepatic and intestinal metabolizing enzymes has not been fully investigated. Like many teleost genes, two VDR genes (VDRα and VDRβ) are observed in some fish species. In concordance with subfunctional portioning theory (Postlethwait et al., 2004), the tissue distributions of the VDRα and VDRβ forms are quite different. In pufferfish, VDRα exhibits a ubiquitous tissue distribution, including strong expression in brain, gill, heart, and to a lesser extent liver (Maglich et al., 2003). Pufferfish VDRβ, on the other hand, is localized solely in the gut. The distribution of both VDRα and VDRβ in the Japanese flounder is widespread, with high expression levels in the gill, brain,

heart, ovary, testis, and gut (Suzuki et al., 2000). Flounder VDRβ expression was found to be weak in liver, whereas VDRα was absent. In both species, the strong expression in the intestine and gut signifies a putative role in regulation of intestinal metabolic activities.

The presence of a high-affinity VDR in the sea lamprey suggests that regulation of calcium is not a critical function of ancestral VDR (Whitfield et al., 2003). Supporting this claim is the observation that VDR expression precedes bone formation during *Xenopus* development (Li et al., 1997). Furthermore, in mammals, VDRs mediate critical functions other than calcium and phosphate regulation, including immune function, skin development, cell proliferation, and catabolism of endogenous and exogenous substrates. The latter two are of particular importance in this consideration of liver toxicology. The fact that lamprey VDR is capable of binding and transcriptional activation of mammalian CYP3A4 promoter demonstrates conservation in the DNA binding behavior of an early form of this receptor and a possible role in endobiotic and xenobiotic metabolism in the livers of fish (Whitfield et al., 2003). More functional data are needed regarding the role of VDR in endobioitc and xenobiotic metabolism. Interesting, like PXR, VDR contains a large insert between helices 1 and 3. While significantly different in sequence than PXR, this insert may be associated with novel low-affinity ligand binding/transactivation activities of VDR, specifically given the differing physical and nutritional environments between aquatic and terrestrial species.

Peroxisome Proliferator-Activated Receptor (PPAR) in Fish

Peroxisome proliferator-activated receptors are ligand-dependent transcription factors belonging to the NR1C subfamily of nuclear receptors. PPARs typically bind natural and synthetic fatty acids and certain pharmaceutical ligands. More recently, however, it has been demonstrated that select members of the PPAR subfamily bind environmental contaminants such as phthalate monoesters and organotins, making them putative mechanistic targets for endocrine disruption (Grun and Blumberg, 2006; Hurst and Waxman, 2004). PPAR nuclear receptors exist in three forms: PPARα, PPARβ, and PPARγ. The ligand-binding affinities for each form differ, as does the tissue-specific expression (Lee et al., 1995, 2003; Schoonjans et al., 1996). In teleosts, all homologs of all three mammalian-defined PPAR isotypes (α, β, and γ) have been identified in both marine and freshwater fish, although differences in total number of PPAR genes (variants of the three isotypes) and their tissue distribution exist in comparison to mammals (Andersen et al., 2000; Batista-Pinto et al., 2005; Ibabe et al., 2002; Leaver et al., 2005; Maglich et al., 2003; Robinson-Rechavi and Laudet, 2001). From the molecular perspective, teleost PPARs heterodimerize with RXR and bind to peroxisomal proliferator response element (PPRE)-like direct repeat elements in promoter/enhancer regions, resulting in altered expression of target genes similar to that observed in mammals (Leaver et al., 2005). Structurally, the *N*-terminal A/B domains of the PPARs are the least conserved regions between fish and mammals; however, DNA-binding domains and ligand-binding domains demonstrate a high degree of sequence identities among fish, amphibians, and mammals, although the LBDs are generally longer in fish (Andersen et al., 2000; Leaver et al., 2005). PPARγ from pufferfish, salmon, flounder, and flatfish are unique in that they contain key amino acid differences within the ligand-binding domains, thus preventing the binding of acidic ligands. It has been suggested that fatty acids are not likely to activate fish PPARγ (Leaver et al., 2005; Maglich et al., 2003). This represents a significant difference between teleostean and mammalian forms of this receptor and is perhaps reflective of early adaptations of the receptor to specific demands present in aquatic environments.

In mammals (rat and mouse), PPARα activation leads to peroxisome proliferation (number and volume) and concurrent transcriptional activation of genes involved in lipid homeostasis. Genes involved in fatty acid uptake and binding (fatty acid binding protein [FABP]), fatty acid ω-oxidation (acyl-CoA oxidase [AOX]), lipoprotein assembly and transport (various apolipoproteins), and others are all modulated by PPARα (Desvergne and Wahli, 1999; Hamadeh et al., 2002; Martin et al., 1997; Schoonjans et al., 1996; Yadetie et al., 2003) and thus are excellent targets of investigation in fish model systems. Although some studies have suggested that fish are refractory to peroxisome proliferation (Baldwin et al., 1993; Pretti et al., 1999), *in vivo* and *in vitro* experiments show that many fish species are responsive to PPARα ligands such as the fibrate-based pharmaceuticals and other natural peroxisome proliferators (Donohue et al., 1993; Haasch et al., 1998; Ruyter et al., 1997; Scarano et al., 1994; Yang et al., 1990). More

recent studies suggest that peroxisome proliferation and AOX expression (typical PPARα-induced liver responses) may be used as biomarkers in aquatic organisms (Cajaraville et al., 2003). Interestingly, however, as with gene regulation of CYP2, little evidence suggests that PPARα activation results in altered expression of CYP4, a common PPARα target in mammals. This again represents a significant departure in CYP gene regulation between teleosts and mammals. Although CYP4 has been identified in several fish species (either by direct cloning or by genome analysis), the mechanisms of CYP gene regulation in teleosts have not been well investigated. Initial similarities between AhR and CYP1A activation suggested that disparate species would share common mechanisms; however, recent observations reveal a lack of induction of CYP4 and CYP2 genes in addition to the lack of CAR. CYP gene regulation in teleosts may be quite different from that of their mammalian counterparts. Moreover, given the dynamic diversity of teleosts and their diverse habitats, significant differences may be observed in the spectrum of NR ligands, binding affinities, and mechanisms regulating gene transactivation.

Role of Nuclear Receptors in Bile Acid, Lipid, and Cholesterol Homeostasis

Multiple NRs play pivotal roles in the regulation of cholesterol, lipids, and bile acid in the livers of mammals. In keeping with the theme that NRs serve as cellular sensors for nutrients and their metabolites, it is now understood that the effects of many dietary compounds, including cholesterol, fatty acids, and fat-soluble vitamins, and other lipids, are mediated by the action of NR binding. Nutritional lipid intake constitutes an important determinant of disease susceptibility and is often exacerbated by dislipidemia. Numerous studies are now addressing questions of the influence of dietary lipids on gene regulation and subsequent changes in metabolism with a goal of establishing how diets can be modified to improve health. Identifying and understanding the activity of nuclear receptors as cellular sensors of cholesterol, lipids, and bile acids and thus their regulation are the subjects of intense study. Cholesterol is essential for all vertebrate cells and is required for maintenance of membrane fluidity and permeability, as a precursor for steroid biosynthesis, and for bile acid production. Homeostatic control of lipoprotein cholesterol uptake and synthesis is regulated at multiple checkpoints through a series of negative feedback loops that respond to elevated intracellular cholesterol levels. These checkpoints are governed by a family of membrane-bound transcription factors—sterol regulatory element binding proteins (SREBPs)—which activate expression of genes involved in the synthesis and uptake of cholesterol and lipogenesis (Horton et al., 2002). To prevent cytoxicity due to cholesterol, oxysterol, and bile acid accumulation, liver X receptor (LXR), farnesol X receptor (FXR), and additional NRs (PXR, LHR, PPAR, HNF4, and SHP) promote sterol storage, transport, and catabolism (Ory, 2004). Key to the coordinated function of these receptors is their role as cellular sensors for lipids, sterols, and bile acids (i.e., PPAR, fatty acids; LXR, oxysterols; FXR, bile acids; PXR/CAR, bile acids). Receptor ligand binding of NRs initiates specific transcriptional programs that regulate sterol homeostasis, including cholesterol uptake and catabolism, triglyceride metabolism, reverse cholesterol transport, lipoprotein biosynthesis, and bile acid synthesis, transport, and metabolism (Chiang, 2004; Ory, 2004).

Completion of genome projects in certain teleosts such as pufferfish, medaka, and zebrafish demonstrated the presence of LXR, FXR, and other NRs associated with cholesterol and lipid homeostasis. These results suggested a similar repertoire of NR function in lower vertebrates. To date, however, information regarding the functional mechanism of NRs in sterol homeostasis in teleosts and other aquatic organisms is lacking and can only be inferred via gene homologies. Identification of many of the genes regulated by these NRs including CYPs, apolipoproteins, and various transporters, however, has provided the additional information that teleosts share a common mechanism of sterol homeostasis with mammalian liver.

Summary

Nuclear receptors are ligand-dependent transcription factors that bind to lipophilic signaling molecules, resulting in the control and expression of target genes in liver and often other tissues. They facilitate the cellular response to endogenous and exogenous ligands by coordinating complex transcriptional responses; therefore, they must be considered in the study of toxicity. Analysis of the evolution, molecular

behavior, and physiological function of NRs is of particular interest given their diverse role in coordinating numerous biological processes. The origins of nuclear receptor interactions are unknown, yet NRs have been found in almost all animal species examined thus far. NRs play a significant role in the liver physiology of all animals, including the uptake, metabolism, storage, and redistribution of nutrients and endogenous molecules, metabolism of xenobiotics, and formation and excretion of bile. Studies in teleosts to date suggest that there are substantial differences in the structure and possibly functions of teleost NRs. Because fish stand between invertebrates and higher vertebrates in evolution, they may serve as unique models for the study of the evolutionary and physiological significance of NRs. From a toxicological perspective, we have yet to determine if these NRs are true targets for exogenous compounds and xenobiotics as described for mammalian species. With PXR, numerous species-specific ligand-binding profiles exist, but the molecular and physiological functions of teleost NRs have yet to be determined. As we continue to explore the molecular dynamics of teleost nuclear hormone receptors and analyze ligand-specific interactions, DNA-binding characteristics, and comodulator recruitment, we will gain new insights into the evolution of NR-mediated transcription regulation and the importance of receptor subfunctionaliztion and will broaden our understanding of the role of NRs in governing liver-specific processes in lower vertebrates.

Introduction to Bile Synthesis and Transport

Bile acid synthesis and secretion, performed by hepatocytes and biliary epithelial cells, are vital for the assimilation of lipid-soluble dietary nutrients (e.g., vitamins A, K, and E; triacylglycerols) and the elimination of endogenous metabolic byproducts and wastes (e.g., bilirubin, hormones). Equally important is the role of bile in the elimination of xenobiotics (environmental toxicants, carcinogens, drugs, and their metabolites). The physiological importance of these processes renders bile synthesis and secretion critical life functions.

The hepatobiliary transport of substances (e.g., bile salts, inorganic and organic solutes) from blood to bile includes three major steps. First, uptake of substances from blood plasma across the sinusoidal membrane and then into the hepatocytes must occur. This in turn is followed by intracellular transport and metabolism. Finally, transport of the parent substances or metabolites occurs across the apical membrane of hepatocytes into biliary passageways (Groothuis and Meijer, 1996; Nathanson and Boyer, 1991).

As a brief review of the biliary passageways in liver, the terminal branches of the intrahepatic biliary system are canaliculi, microscopic passageways 1 to 2 mm in diameter, that are formed by, and between, adjacent hepatocyte membranes and delineated by tight junctions. Next are transitional zones in which bile preductular epithelial cells (cells with high nuclear-to-cytoplasmic ratios) form junctional complexes with hepatocytes, at which are created bile preductules. Bile preductules provide for movement of bile from canaliculi to bile ductules (cholangioles). The walls of the latter are completely comprised of biliary epithelial cells. From here, bile passes through a network of ductules that feed larger intrahepatic bile ducts as the bile moves toward the liver hilus. From the hilus, bile is conducted to the extrahepatic biliary system. The latter is comprised of one or more hepatic ducts that conduct bile into the bile duct. From this structure, a cystic duct arises that conducts bile to and from the gallbladder. From the point between the cystic duct and the intestinal wall, the passageway is known as the common bile duct. All of these components are discussed in the section on cells of the teleost liver.

Because pressures within bile spaces exceed those of the sinusoids, hydrostatic forces alone cannot account for bile flow (Trauner and Boyer, 2003). Flow of bile from sinusoids to canaliculi (blood to bile transport), through the hepatocyte, is a secretory and concentrative process driven by a suite of transmembrane transporters (discussed later in this chapter); for example, in rodents blood to bile transport is uphill against a 100- to 1000-fold concentration gradient. Bile flow in the intrahepatic biliary system is also regulated by the contraction of canaliculi, the functional aspects of which are mechanically governed by cytoskeletal elements in the pericanalicular cytoplasm. Because cytoskeletal proteins are key regulators of proper canalicular development and function, impairment of cytoskeletal function by endogenous or exogenous compounds is a key mechanism by which altered bile transport (toxicity)

occurs. In the mammalian liver, altered bile transport (cholestasis) is a common response of the liver to xenobiotic exposure and a key mechanism of toxicity for many drugs. Changes to cytoskeletal function, for example, are associated with a loss of canalicular microvilli and diminished canalicular contractility (Phillips et al., 1986; Song et al., 1998; Watanabe et al., 1983).

In the intrahepatic biliary system of mammals, biliary epithelial cells (BECs), like hepatocytes, are key regulators of bile transport, responsible for regulating: bile fluid alkalinity (through the Na^+-dependent Na^+/HCO_3^- and CL^-/HCO_3^- exchange symporters), electrolyte content (via ion channels), water composition (a major components of bile, via active transport of aquaporins), and bile salts. Bile salt uptake into cholangiocytes and gallbladder epithelial cells may also serve an important role via cell signaling for regulation of secretory and proliferative processes (in response to injury) within the biliary tree (Alpini et al., 2001, 2002).

Biliary epithelial cells (BECs) are known target cells in a number of pathologic conditions (cholangiopathies) of mammalian liver, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), as well as diseases associated with BEC proliferation or loss. For example, in rodents, cholangiopathies that result in impaired bile synthesis or flow can be induced by several experimental conditions, such as chronic administration of CCl_4 or the reference toxicant α-naphthylisothiocyanate (ANIT) (Kanno et al., 2001). Because of the critical role of BECs in bile homeostasis, impairment of BEC function can be a primary source of hepatotoxicity through altered bile synthesis and transport. Although BECs have been identified in several fish species and their morphology characterized, their complex transport and bile regulatory mechanisms remain poorly understood. It is likely, however, that their functional characteristics are equally important in the hepatobiliary toxicity of fishes.

Bile Synthesis

Hepatocytes and biliary epithelial cells coregulate bile synthesis, volume, composition, and transport in response to changing physiologic needs. In all vertebrates, bile synthesis is part of the mechanism of cholesterol elimination, accomplished by conversion of cholesterol to water-soluble amphipathic bile salts (Moschetta et al., 2005). In higher vertebrates, metabolic conversion of cholesterol to the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) occurs in hepatocytes via two pathways. The classic (neutral) synthetic pathway involves modification of the sterol nucleus (e.g., 7α-hydroxylation and β-oxidations of the side chain of either cholesterol or one of three oxysterols containing a hydroxyl group at the C24, C25, or C27 position of the side chain) by 7α-hydroxylase (CYP7A1), leading to formation of CA and CDCA. The acidic, or alternative, pathway is catalyzed by sterol 27-hydroxylase (CYP27A1) located on inner mitochondrial membranes, leading to the generation of CDCA. In both processes, the end products CA and CDCA are subsequently amidated to anionic bile salts (bile acids exist as bile salts at physiological pH) via glycine or taurine conjugation, rendering them impermeable to cell membranes and hydrophilic. During enterohepatic transport, CA and CDCA are metabolized by intestinal flora (bacteria) to the secondary bile acids deoxycholic acid (DCA; from CA) and lithocholic acid (LCA; from CDCA).

In primitive vertebrates, including fish, bile synthesis pathways consist of different means of hydroxylating cholesterol, followed by conjugation with a strong anion (usually sulfate). The most common products in lower vertebrates are bile alcohol sulfates with four, five, or six hydroxyl groups per sterol nucleus. It is presumed that, through evolution, bile salts gradually assumed more diverse biological roles, reflected in structural variations (lipid solubilization, bactericidal). One of the more significant evolutionary advancements in bile synthesis was the shift from nonrecycling C27 bile alcohol sulfates (e.g., fish and reptiles) to enterohepatically cycling C24 bile acids. C24 bile acid structures have been achieved in virtually all vertebrate groups through convergence, with each group achieving the C24 bile acid structure independently. A survey of the transition in bile synthesis in various species found three primary evolutionary transitions: (1) C27 alcohol sulfates to C24 taurine-conjugated acids, (2) C27 alcohol sulfates to C27 taurine-conjugated acids to C24 taurine-conjugated acids, and (3) C27 alcohol sulfates to C24 glycine-conjugated acids to C24 taurine-conjugated acids. The first pathway is considered quite rare and has been observed only in fish. The second pathway is the classical, most commonly used route in lower vertebrates, and the third pathway is the one utilized in mammals. Vertebrate groups in

which species have evolved over a long enough period of geological time have members that form C27 bile alcohol sulfates, species that are in the process of transition (selecting one of the three pathways), and species that form C24 taurine-conjugated acids. Hence, bile acids in vertebrates can be broken down into three classes based on side-chain functional group: C27 bile alcohols and C27 and C24 bile acids. Bile alcohols, which occur in many fish species, are conjugated by esterification of the terminal C27 hydroxy group with sulfate, whereas bile acids are typically conjugated by *N*-acyl amidation of the terminal C27 or C24 carboxyl group with taurine or glycine (Moschetta et al., 2005).

In terms of bile synthesis and composition, the majority of fish fall into two groups: ancient (utilizing C27 bile alcohol sulfates) and modern (utilizing C24 taurine-conjugated acids). C27 bile alcohol sulfates, characteristic of a more primitive bile synthetic pathway, are ubiquitous among vertebrates. They are the dominant bile salts of ancient mammals (e.g., elephant, manatee, rhinoceros) and the major bile constituents in cartilaginous fish, herbivorous bony fish, and some amphibians. In the few fish species examined, the ratios of bile acids to bile alcohols differ from those of mammals; for example, medaka synthesize bile using the classical pathway (a transition fish), with approximately 50% C27 taurine-conjugated acids and 25% C24 taurine-conjugated acids, while a sulfated bile alcohol, scymnol sulfate (ScyS), is the major bile salt in the little skate (*Raja erinacea*), and 5-cyprinol sulfate, a more common piscine bile alcohol, is the major bile salt in zebrafish. Similar differences in bile acid/bile alcohol composition are described in the coelacanth (*Latimeria chalumnae*) and Japanese eel (Kihira et al., 1984; Masui et al., 1967). Of interest is that bile alcohols are known to be stable at 4°C, which may be of evolutionary significance in cold-water species.

Bile Transport

The vast majority of the bile salt pool is localized to enterohepatic circulation and is regulated in mammals by a suite of distinct transmembrane transporters in hepatocytes, BECs, and enterocytes (Trauner and Boyer, 2003). Bile salt homeostasis is also governed through tight negative feedback control via transcriptional regulation; for example, after intestinal absorption bile salts return to liver where they regulate their own synthesis (see discussion on nuclear regulators). Hepatobiliary transport mechanisms determining uptake and excretion of bile salts and other biliary constituents in liver and extrahepatic tissues are well characterized in mammals and are likely well conserved across vertebrate species (with anticipated subtle species differences). Transmembrane transport proteins, localized to both sinusoidal and apical membranes of hepatocytes and biliary epithelia, have been functionally characterized, cloned, and studied in transgenic animal models, and several genes regulating transporter synthesis have been identified through mutations in inherited forms of cholestasis (Boyer, 1996; Pauli-Magnus and Meier, 2005; Trauner and Boyer, 2003).

The primary mammalian hepatocytic transporters include the sinusoidal Na^+ taurocholate cotransporting polypeptide (NTCP; SLC10A1), organic anion-transporting polypeptides (OATPs; SLC21A), the organic cation family of transporters (OCTs), multidrug resistance-associated proteins (MRP1–6; ABCC1–6), and members of the family of multidrug resistance P-glycoproteins, such as MDR1 (ABCB1), MDR3 (ABCB4), and the apical bile salt export pump (BSEP) (ABCB11) (Boyer, 1996; Groothuis and Meijer, 1996; Pauli-Magnus and Meier 2005; Sturm et al., 2001b; Trauner and Boyer 2003). Bile salt transporters have also been identified in biliary epithelia, enterocytes, the renal proximal tubule of the kidney, and placenta. An important characteristic of MDR-encoded gene products (e.g., MDR1, MDR3) is their broad specificity; they are able to transport an array of structurally and functionally diverse substrates, including anticancer drugs such as paclitaxel, vinca alkaloids, anthracyclines, and epipodophyllotoxins (Ueda et al., 1997), as well as numerous other classes of compounds such as calcium channel blockers, HIV protease inhibitors, hormones, pesticides, cyclic and linear peptides, and immunosuppressive agents (Stouch and Gudmundsson, 2002).

Mammalian bile salt transport occurs via both Na^+-dependent and Na^+-independent mechanisms. The primary Na^+-dependent transporter is the sinusoidal NTCP (Ntcp in rodents), which is exclusively expressed in hepatocytes and mediates the hepatocellular uptake of bile salts (Fardel et al., 2002; Groothuis and Meijer, 1996; Lecureur et al., 2000). In addition to bile salts, rat Nctp has been shown to support the hepatic uptake of estrone-3-sulfate, dehydroepiandrosterone sulfate, and the thyroid

hormones T_3 and T_4. Sodium-independent transport is regulated by the adenosine triphosphate (ATP)-dependent superfamily of transporters (e.g., MDR1/ABCB1, MDR3/ABCB4, MRP2/ABCC2, BSEP/ABCB11) and the multispecific organic anion and cation transporters (e.g., OATPs/SLC21A, OCTs). Of these, MRP2, which transports primarily glutathione and glucuronyl conjugates, including the physiologically important conjugates of bilirubin, estradiol, and xenobiotic metabolites (Faber et al., 2003; Keppler et al., 1997), is the major determinant of bile-salt-independent bile flow and the only known MRP-related canalicular transporter.

Expression of transporter proteins is regulated by multiple and complex biofeedback mechanisms, involving gastrointestinal hormones and peptides such as secretin, vasoactive intestinal peptide (VIP), bile salts within the bile pool, intermediate bile acid metabolites, nuclear receptors, and the cholinergic nervous system (Boyer 1996; Trauner and Boyer 2003; Trauner et al., 2000). Bile acids and salts themselves function as specific ligands for nuclear hormone receptors where they regulate transporter expression via transcriptional events (Chawla et al., 2001).

Fish Studies

Although we currently have only a nascent understanding of piscine hepatobiliary transport mechanisms, it is becoming clear that certain transport mechanisms may be conserved in vertebrate evolution; for example, basolateral uptake of bile acids by trout hepatocytes, as well as the disruption of these transport processes by toxicants, has been demonstrated (Rabergh et al., 1992). Likewise, a sodium-independent carrier system that mediates taurocholic acid and cholic acid transport has been identified in trout (Rabergh et al., 1994). Hepatocellular uptake of bile acids in trout was found to resemble corresponding systems in mammalian liver cells, although trout carriers were distinguished by high efficiency at low temperatures. Below is a comparative summary of known transporters in fish.

MDR-Encoded Transporters

Several highly conserved members of the MDR1 (P-glycoprotein [Pgp]) transporter family have been identified in rainbow trout (Sturm et al., 2001b) and winter flounder (*Pleuronectes americanus*) (Chan et al., 1992). Immunohistochemical studies have also shown conserved distribution of Pgp epitopes in the tissues of guppy (*Poecilia reticulata*), epitopes that resemble mammalian forms (e.g., react with mammalian antibodies) (Hemmer et al., 1998). Other studies have identified MDR-like proteins. Localization experiments using immunohistochemical techniques with an antibody prepared against human Pgp revealed positive reaction at the bile canalicular pole in *Fundulus heteroclitus* hepatocyte cultures (Albertus and Laine, 2001). Similarly, a Pgp-related protein of approximately 170 kDa was demonstrated in the intestine and liver of catfish (Doi et al., 2001). Several studies using isolated fish hepatocytes have demonstrated transport activities exhibiting characteristic Pgp-mediated transport (Albertus and Laine, 2001; Sturm et al., 2001b; Tutundjian et al., 2002). Pgp reversal agents, for example, have been shown to preclude the transport of Pgp substrates in teleost hepatocytes. In rainbow trout hepatocytes, both the accumulation and efflux of the fluorescent Pgp substrate (rhodamine 123) were modified by the reversal agents verapamil, cyclosporin A, and vinblastine, as well as the ATPase inhibitor vanadate (Sturm et al., 2001b). In contrast, tetraethylammonium chloride, a substrate for type I sinusoidal organic cation uptake systems and electroneutral canalicular H^+/organic cation antiporter, had no effect on rhodamine 123 transport (Sturm et al., 2001b). *In vitro* preparations of isolated killifish and turbot hepatocytes and *in vivo* studies in carp and catfish have demonstrated the uptake of fluorescent Pgp substrates that proved to be verapamil and estradiol (E2) sensitive (Albertus and Laine, 2001; Kleinow et al., 2004; Smital and Sauerborn, 2002).

Organic Anion-Transporting Polypeptides (OATPs)

Organic anion-transporting polypeptides have been cloned and functionally characterized in the skate (*Raja erinacea*) (Cai et al., 2002). Phylogenetic and sequence comparisons with a liver-specific OATP isolated from hepatocytes indicate that skate OATP is most closely related to OATP-F (SLC21A14) from human brain (50.4% identity) and rat OATP4, which is also called Lst1 (Slc21a10; 41.2% identity) (Cai

et al., 2002). A multispecific organic solute and steroid transporter that requires the coexpression of two gene products, Ostα and Ostβ, has also been isolated and functionally characterized in skate. The cDNA and predicted amino acid sequences of Ostα and Ostβ appear to be novel and are likely to be members of an additional family of transporters. The overall substrate specificity of Ostα and Ostβ is most similar to that of the OATP family, in particular to that of human OATP-C (also known as LST1 and OATP-2) (Wang et al., 2001). Jacquemin et al. (1995) also identified an organic anion transport protein in skate liver, and these studies suggest that the primitive skate OATP is most closely related to humans.

Multidrug Resistance-Associated Proteins (MRPs)

Genes for MRPs have been identified in red mullet (*Mullus barbatus*) (Sauerborn et al., 2004), and an MRP2 ortholog has been characterized in the liver of the small skate (*Raja erinacea*) (Cai et al., 2003). Antibodies directed against mammalian MRP2-specific epitopes labeled a 180-kDa protein band in skate liver plasma membranes and stained canaliculi by immunofluorescence, indicating that skate livers expressed a homologous protein (Rebbeor et al., 2000). Investigations of ATP-dependent transport in skate liver plasma membrane vesicles suggest that the transport of anions is mediated by a functional analog of mammalian MRP.

Functional and Structural Evidence for a BSEP/SPGP

A BSEP-like protein in the canalicular membrane of the skate liver has been identified and shown to transport both bile salts and bile alcohols (Ballatori et al., 2000). A BSEP-like protein has also been identified in medaka (*Oryzias latipes*) (Hinton et al., unpublished studies).

Bile Flow

Bile flow rates, perhaps expectedly, vary between piscine and mammalian species. Although few aquatic species have been studied, bile flow information for some cartilaginous fish and teleosts exists. For comparative purposes, in humans bile acid secretion by the hepatocyte approximates 1 g/hr, 95% of which is recovered following absorption from the distal ileum into the portal venous system (Wolkoff and Cohen, 2003). The human bile salt pool size is approximately 50 to 60 μ*M*/kg body weight and averages 3 to 4 g (Trauner and Boyer 2003). In humans, bile salts are rapidly extracted from enterohepatic circulation by the liver with single-pass extraction rates as high as 80%. Na^+-dependent uptake of a variety of bile salts is considered to be the rate-limiting step in bile-acid-dependent bile flow, a mechanism that finds a Na^+/bile acid cotransport with stoichiometry of >1:1. After common bile duct ligation and cannulation, both the spiny dogfish shark (*Squalus acanthias*) and the skate (*Raja erinacea*) were observed to secrete bile for periods of 4 to 7 days with maximum rates of 1.77 ± .89 mL/kg per 24 hours in *Squalus acanthias* (approximately 100 times slower than rat) and 2.66 ± .89 mL/kg per 24 hours in *Raja erinacea* (Boyer et al., 1976a,b). Other reported bile flow rates are 33 to 74 μL/kg/hr for *Squalus acanthias* and 75 to 111 μL/kg/hr for *Raja erinacea* (Klaassen and Watkins, 1984). Bile flow in the common bile duct of cannulated rainbow trout (*Oncorhynchus mykiss*) was observed to be constant at 75 μL/kg/hr over 108 hours of experimentation (Grosell et al., 2000). The bile acid concentration in the hepatic bile of rainbow trout (15 to 50 m*M*) is within the range reported for mammals (Klaassen and Watkins, 1984; Strange 1984). A comparison of bile composition and bile flow rates can be found in Table 7.6.

Reported bile flow rates are known to vary among vertebrate species, with aquatic species showing slower rates of bile flow. Where flow rates vary markedly, the fundamental composition of bile, or bile fluid, can be similar, as can be seen in freshwater trout, rats, and humans. Saltwater fish show approximately twice the concentration of electrolytes (Na, K, Cl). Although sparse information is available for aquatic species, current data generally show that bile synthesis and transport are fairly well-conserved physiological functions; for example, skate BSEP clones were identified that share approximately 65% identity with human BSEP. Other homologous mammalian transporters identified in fish are Pgp, OATP, MRP, and NTCP. Similarly, the human nuclear receptors FXR, LXR, CAR, PXR, VDR, and PPARα, -β, and -γ have also been identified in piscine species. Given our increasing understanding of conserved

TABLE 7.6

Comparative Bile Composition Among Vertebrates

	Trout[a]	Rat[b]	Human[c]	Skate[d]	Dogfish[d]
Na	152 m*M*	158 m*M*	130 m*M*	295 m*M*	271/366 m*M*
K	—	6.3 m*M*	5 m*M*	4.6 m*M*	5–6.5 m*M*
Cl	134 m*M*	99 m*M*	100 m*M*	221 m*M*	224 m*M*
CO_2/HCO_3^-	7.9 m*M*	20 mEq/L HCO_3^-	30 m*M*	5/6 m*M* HCO_3^-	5.8 m*M*
Bilirubin	—	—	15 mmol/mol	—	—
Bile acids	<20 μ*M*	15.9 μ*M*	9–120 mmol/mL	7.9 μ*M*	21 m*M*
	—	1.39 μEq/L/kg	—	—	—
Osmolarity	297 mOsmol	302 mOsmol/L	—	—	922 mOsm
Cholesterol	—	0.37 mEq/L	10 mmol/L	—	—
Flow rate	75 μL/kg/hr over 108 hr	85.6 μL/min/kg	500–800 mL/d	~2 mL/kg body wt per 24 hr	~2 mL/kg body wt per 24 hr
	—	2.25 μL/min/g	—	2.66 ± .89 mL/kg per 24 hr	1.77 ± .89 mL/kg per 24 hr

[a] Data from Grosell, M. et al., *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, 278(6), R1674–R1684, 2000.
[b] Data from Alpini, G.R. et al., *J. Clin. Invest.*, 81(2), 569–578, 1988.
[c] Data from Hoffman, A.F., in *Physiology of the Gastrointestinal Tract*, Johnson, L.R., Ed., Raven Press, New York, 1994.
[d] Data from Boyer, J.L. et al., *Am. J. Physiol.*, 230(4), 970–981, 1976.

bile synthesis and transport physiology across vertebrates, it is interesting to consider the future of small fish animal models, such as STII medaka, as surrogates for human hepatobiliary transport study.

In summary, it is becoming increasingly clear that the mechanisms involved in the regulation of bile acid synthesis, lipid metabolism, and hepatobiliary transport in vertebrates are highly integrated and, not surprisingly, involve a complex biofeedback network composed of bile salts, nuclear receptors, transcription factors, and a variety of intermediate signaling proteins. The majority of currently known mechanisms have been elucidated in rodent and human studies, and, although information for piscine species remains sparse, it is increasingly apparent that many bile synthesis and regulatory mechanisms are conserved across vertebrate species.

Biliary System Toxicity: Mechanistic Considerations

During cholestasis in mammals, bile salt transport proteins undergo adaptive responses that serve to protect the body from bile salt retention and maintain homeostasis (Trauner et al., 2005). When adaptive responses of the hepatobiliary system are precluded by xenobiotic-induced alterations to hepatobiliary structure and function, impairment of bile transport may be a major source of toxicity, either through the direct action of toxic bile salts on cell function or the toxicity resulting from impaired elimination of xenobiotics and their metabolites from within the cell or systemic circulation. Cholestasis (impairment of bile synthesis and transport) results in mammals due to exposure to a wide range of drugs and other xenobiotics and is seen in genetic disorders regarding bile synthesis and transport. The condition has received abundant attention in mammals (Anwer, 2004; Arrese and Trauner, 2003; Muller and Jansen, 1998; Trauner et al., 1998), but much less so in fish. Cholestasis is important for several reasons: (1) the formation and secretion of bile are essential for life, (2) bile synthesis and transport are among the most easily disrupted liver functions, and (3) for most cholestatic agents, the exact mechanism of xenobiotic-induced cholestasis remains unknown (Faber et al., 2003). The cholestatic response is more thoroughly understood in the mammalian liver due to investigations on the adverse effects of altered bile synthesis and transport in humans and the extensive use of rodent models in cholestasis-related research.

Cholestasis is most commonly not fatal, although various pathologic sequelae are associated with this condition. Chronic manifestations of cholestasis include systemic toxemia, impaired immune function, and central nervous system and renal system stress. Hence, failure or impairment of bile excretory mechanisms can lead to general conditions of systemic toxemia.

Bile-acid-induced toxicity, which may result in cholestasis, is often associated with perturbation of mitochondrial respiration and depolarization (MPT) and cell death (Aguilar et al., 1996; Benz et al., 2000; Palmeira and Rolo, 2004; Rolo et al., 2004). Increasing concentrations of the bile acids LCA, DCA, UDCA, CDCA, glycochenodeoxycholic (GCDC), and taurochenodeoxycholic (TCDC) were shown to decrease mitochondrial membrane potential, decrease state 3 respiration, and enhance state 4 respiration (Rolo et al., 2004). Bile salts themselves have been observed to alter energy-dependent transport dynamics via modulation of normal cellular energy production and regulation. In short, impairment of bile transport via compromise of hepatocyte or cholangiocyte function is a major source of toxicity, from both endogenous substances and xenobiotics.

Alterations to cytoskeletal and gap- and tight-junction proteins are primary mechanisms by which cholangiopathies may occur, and they are seen following exposure to xenobiotics. The naturally occurring toxins microcystin-LR (MC-LR) and okadaic acid (OA), for example, inhibit cytoskeletal function via inhibition of dephosphorylation, and microcystin, a protein phosphorylation (PP2A) inhibitor, is known to induce cholestasis in humans via altered microtubule-dependent vesicle movement in hepatocytes (Pouria et al., 1998). OA, which also blocks PP2A, inhibits cAMP stimulation of TC uptake, translocation, and dephosphorylation of NTCP and increases cytosolic Ca^{2+} (Mukhopadhyay et al., 1998). Similarly, administration of phalloidin leads to rapid and sustained cholestasis in rats through cytoskeletal changes resulting in impaired canalicular function. These changes were linked with a total loss of MRP2 function (possibly due to altered translocation of the protein from the Golgi apparatus to the outer cell membrane) (Rost et al., 1999). Phalloidin damages cytoskeletal function and arrangement and results in sustained cholestasis in rats through impairment of canalicular function and a total loss of MRP2 expression (Rost et al., 1999).

Toxicants such as α-naphthylisothiocyanate (ANIT) are also known to alter cytoskeletal function and induce cholestasis in rats (Lesage et al., 2001; Orsler et al., 1999; Palmeira et al., 2003), and preliminary studies in the Hinton laboratory have shown that ANIT targets both hepatocytes and biliary epithelia in medaka (Hinton, unpublished studies).

Related Studies of Biliary Toxicity in Livers of Fishes

A variety of diseases related to biliary dysfunction such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are well recognized in mammals, but hepatobiliary-related disease states, as well as impaired transport function, are only beginning to be understood in fish species (Cai et al., 2001; Kirby et al., 1995; Lorent et al., 2004). Studies in cartilaginous fishes provide evidence for cytoskeletal-related impairment to hepatobiliary transport. They have generally employed clusters of isolated polarized skate hepatocytes, and they have provided structural and functional evidence for microtubule-dependent transcytosis. As an example, the polarized arrangement of microtubules, presence of cytoplasmic dynein, and inhibition of bile salt secretion by nocodozole were consistent with the microtubule cytoskeleton playing a fundamental role in the mediation of transcytosis, endocytosis, and bile excretory function in skate hepatocytes (Henson et al., 1995).

Published findings on xenobiotic-induced alterations of P-glycoprotein in fish are conflicting. For example, Sturm et al. (2001a) found no induction of C219-immunoreactive Pgp protein levels after *in vivo* exposure of rainbow trout to prochloraz or nonylphenol diethoxylate. Similarly, dietary exposure of channel catfish to the xenobiotics β-naphthoflavone, benzo(*a*)pyrene, or 3,4,3′,4′-tetrachlorobiphenyl did not result in elevated levels of C219-immunoreactive proteins nor in altered Pgp activity in intestine and liver (Doi et al., 2001). In contrast, the expression of hepatic C219-immunoreactive Pgp protein was significantly enhanced in the liver of killifish, both *in vitro* and *in vivo*, after exposure to chlorpyrifos oxon and the carcinogen *N*-nitrosodiethylamine (Albertus and Laine, 2001).

Complex relations between xenobiotics and Pgp expression in fish are indicated from a study on intertidal blennies (*Anoplarchus purpurescens*) (Bard et al., 2002b). Hepatic Pgp immunostaining with C219 antibody was significantly elevated in blennies collected from field sites downstream of pulp mills,

as compared with fish depurated for more than 4 weeks in clean water in the laboratory; however, in the field-sampled fish, a gradient of hepatic Pgp expression correlating with proximity to the pulp mill effluents was not evident. In laboratory experiments, blennies exposed to oiled sediments exhibited significantly elevated hepatic Pgp levels, while blennies exposed to contaminated sediments from the pulp mill sites or injected with β-naphthoflavone showed no elevation of hepatic Pgp levels.

When a creosote-resistant killifish population from a polluted site in the Elizabeth River in Virginia was studied (Cooper et al., 1999), adult fish showed a high prevalence of hepatic tumors. In immunoblots with the C219 antibody, both normal and neoplastic livers of killifish from the polluted site contained significantly higher levels of Pgp-like proteins than did livers of reference killifish. This finding may be taken as an indication that Pgp contributes to creosote resistance, since biliary excretion is a major elimination route for PAH metabolites.

In contrast, studies on killifish in New Bedford Harbor in Massachusetts (Bard et al., 2002a) revealed an acquired resistance to planar halogenated aromatic hydrocarbons (PHAHs), and the contaminant-resistant fish showed lower levels of hepatic C219-immunoreactive protein than did killifish from a relatively unpolluted site. Interestingly, fish from New Bedford Harbor displayed Pgp-like protein in the intestine, whereas fish from the relatively unpolluted site exhibited no detectable Pgp in the intestine. The authors speculated that fish at New Bedford Harbor are exposed to xenobiotics that are inducers of a gut-specific Pgp isoform and that intestinal uptake is the dominating route of xenobiotics uptake.

The question remains whether elevated levels of hepatic or intestinal Pgp observed in contaminant-resistant killifish from polluted sites reflect an exposure to Pgp inducers. Because fish from the polluted field sites showed strongly declining levels of Pgp expression after culture in clean water (Bard et al., 2002b), the argument can be made for a role of xenobiotics in the induction of Pgp expression; however, results from laboratory exposure experiments that directly demonstrate a role of environmental chemicals in elevating the Pgp expression of killifish are elusive. Exposure of killifish to the PHAH 2,3,7,8-tetrachlorodibenzofuran, known to be present in New Bedford Harbor, did not stimulate intestinal or hepatic Pgp expression (Bard et al., 2002a). Likewise, when Cooper et al. (1999) injected both reference and creosote-resistant killifish with 3-methylcholanthrene, a PAH contained in creosote, they found no increase of hepatic levels of Pgp-like proteins. In summary, based on the current information, contaminant-resistant fish may show altered Pgp expression but the cause-and-effect relationship of xenobiotic exposure lacks verification. Overall, the database on the relation between xenobiotics and Pgp in fish is small and dispersed. Published reports do not yield a consistent pattern, and this may be explained in part by interlaboratory differences in species, xenobiotics, and experimental design; however, it may also be due to technical problems, such as the lack of fish-specific probes for Pgp, and to insufficient knowledge on the basic parameters of Pgp regulation in fish. Pgp expression can be induced as part of a general response to cellular stress, and this may be regulated by ligands of the PXR, thus sharing elements of regulation with an important group of mammalian biotransformation enzymes, CYP3A.

Further, mammalian Pgp and CYP1A share some inducers, albeit they are not regulated by the same mechanisms. For fish, no published evidence exists on PXR, and the understanding of piscine CYP3A, despite some ortholog characterization in teleosts (Kullman and Hinton 2001; Kullman et al., 2000), remains fragmentary. Several studies with fish were attempted to provoke a co-induction of CYP1A and Pgp. When *Fundulus heteroclitus* were exposed to the AhR ligand 3-methylcholanthrene and observed (Cooper et al., 1999), an induction of CYP1A but not of Pgp expression occurred. Similarly, Bard et al. (2002a) exposed killifish to the AhR ligand 2,3,7,8-tetrachlorodibenzofuran with resultant induction of CYP1A and not Pgp. In rainbow trout, prochloraz induced hepatic CYP1A but not hepatic Pgp (Sturm et al., 2001a). A correlation between CYP1A and Pgp levels was found in blennies collected at polluted field sites; however, when blennies were treated in the laboratory with the AhR ligand β-naphthoflavone, only CYP1A (not Pgp) was elevated (Bard et al., 2002b). In flounders collected at three sites with differing pollution levels in Seine Bay (English Channel), CYP1A and Pgp levels showed exactly opposite trends (Minier et al., 2000). These findings suggest that Pgp and CYP1A are not coordinately regulated in fish; however, these two proteins may act in complementary ways in cellular detoxification.

Virtually nothing is known about cholestasis in fish, and there is an urgent need for understanding piscine cholestasis given that the pathogenesis of other toxic alterations in piscine liver is at least qualitatively similar to that of mammalian liver. Two studies in medaka showed that chronic exposures

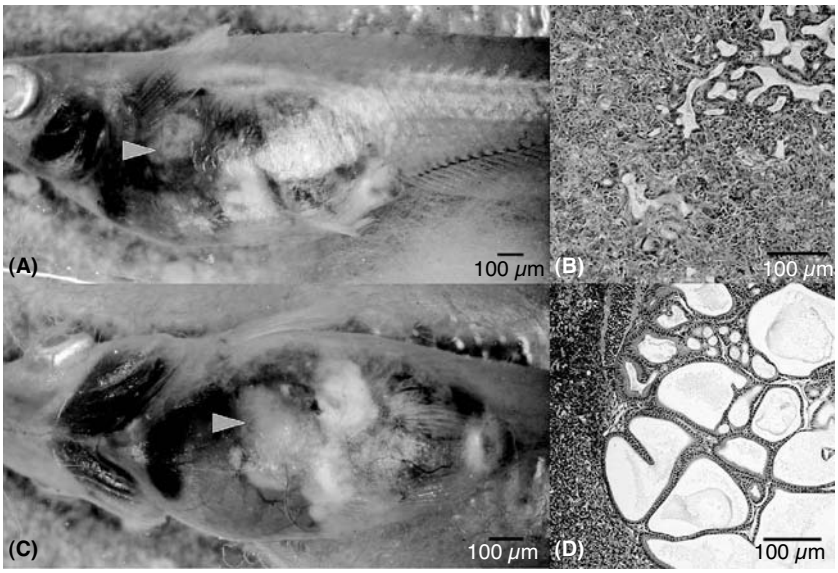

FIGURE 7.16 (See color insert following page 492.) *In vivo* imaging of tumor formation in STII medaka (brightfield microscopy). Neoplastic response following early life stage exposure of STII medaka to the reference hepatocarcinogen diethylnitrosamine (DEN). Aqueous bath exposure was of 24-hour duration at 100 ppm DEN. Fish were exposed as 14-day-old hatchlings and followed serially in a noninvasive manner for 10 months. At 10 months after exposure, a subset of cohorts developed hepatic tumors (green arrowhead). (A, C) *In vivo* imaging (brightfield) of hepatic tumor formation (green arrowheads) in DEN-exposed medaka; shown is an enlargement of the total liver mass, approximately 18% of the total body length. After anesthesia, the liver was removed and processed for histopathology. Hepatic neoplasm showed mixed hepatocellular (B) and cholangiocellular (D) carcinomas. Focus of biliary hyperplasia was seen in the same liver; here, a single layer of biliary epithelium lines large cystic spaces in the liver (D). The opaque white tissue in the brightfield images (A and B) is ovary; the gut occupies caudal most region of the abdominal cavity.

to bromodichloromethane or chloroform induced bile duct dilatation and cystic enlargement of extrahepatic bile passageways (Toussaint et al., 2001a,b). Studies of hepatic carcinogenesis in trout (Hendricks et al., 1984) and in medaka (Okihiro and Hinton, 1999) have also shown that the intrahepatic biliary epithelial cells are involved in chronic toxicity. Cholangiomas, cholangiocellular carcinomas, and mixed hepato- and cholangiocellular carcinomas have been reported (Hinton, 1993b) (Figure 7.16). From this brief review, it is apparent that the biliary system is a target of xenobiotic-induced toxicity in fishes and that better understanding of the involved mechanisms is urgently needed.

Factors Influencing Xenobiotic-Induced Liver Injury

Because of its central location in the circulatory system of vertebrates, the liver is a target for many toxicants, and it receives toxic substances both from intestinal and branchial routes. Further, due to the large exchange surface at the sinusoidal pole of the hepatic parenchymal cells, together with an array of uptake systems and transporters at the hepatocyte membrane, the liver is able to efficiently extract toxicants from the circulation. The toxic chemicals can directly impair liver structures and functions; for example, by inducing cytotoxic damage and necrosis they can induce toxic effects after being metabolized into reactive molecules, or they can act indirectly through affecting interactions and signaling between the various liver cell types. Toxicant action may also impact seemingly unrelated hepatic functions. An example is provided by the antiestrogenic activity of AhR-binding xenobiotics. Induction of the AhR pathway by these compounds is associated with a reduced synthesis of hepatic vitellogenin, which is under the control of estrogen (Anderson, 1996; Navas and Segner, 2000, 2001). This antiestrogenic effect of AhR-binding xenobiotics is mediated through a cross-talk between the AhR and the ER (Cheshenko et al., 2007; Ohtake et al., 2003), thereby resulting in a toxicant effect on an unexpected target.

Cell Interaction

A mechanistic understanding of the mode of action of various xenobiotics is possible only with knowledge of the cell types involved in the activation and deactivation of the compound and those that become targets for toxicants. Current mammalian liver toxicity studies demonstrate signaling between individual cell types that often determines the site and extent of toxicity (Treinen-Moslen, 2001). Hepatotoxicants such as acetaminophen or carbon tetrachloride, for example, can lead to an increased number and activation of macrophages, including the resident macrophages (i.e., Kupffer cells) in the rodent liver (Laskin and Gardner, 1998). Also, the sinusoidal endothelial cells and stellate cells can increase in number and become activated following exposure of rodents to hepatotoxicants. The activation of the sinusoidal cells may occur through factors released by damaged hepatocytes (Treinen-Moslen, 2001). The activated cells release mediators such as reactive oxygen intermediates, reactive nitrogen intermediates, cytokines, bioactive lipids, and hydrolytic enzymes that then are responsible for sequelae reactions (Dong et al., 1998; Ganey et al., 2001; Junge et al., 2001; Morio et al., 2001). In the stellate cells, collagen production is stimulated (Treinen-Moslen, 2001). The mediators released by the nonhepatocyte cells can be directly cytotoxic, but they are also able to modify hepatocyte signaling pathways or xenobiotic metabolism (Cain and Freathy, 2001; Renton, 2000). Perhaps the best evidence for a role of macrophages in the pathogenesis of liver injury comes from studies where the experimental depression of macrophage function diminished hepatotoxicity (Junge et al., 2001; Laskin and Gardner 1998). Few studies have addressed the possible role of nonhepatocyte cells, inflammatory macrophages, and macrophage-derived factors in liver damage in fish.

From studies on the acute phase response of fish, evidence has been obtained that salmon hepatocytes possess cytokine receptors and that bacterial lipopolysaccharide (LPS) induces serum amyloid A transcription in the hepatocytes (Jorgensen et al., 2000); however, whether this response was a direct effect of LPS on the hepatocytes or mediated by other liver cell types was not determined. Pollutant-induced activation of catfish (*Heteropneustes fossilis*) macrophages was associated with a concomitant increase in peroxidative tissue damage (Fatima et al., 2000). The liver, however, showed the weakest response among the three target organs studied (gills, liver, kidney). In mammalian liver, cytokines suppress hepatic cytochrome P450 enzymes (Renton, 2000), and, as reported by Marionnet et al. (1998), they also suppress cytochrome P450 activities in fish liver. Furthermore, while cell types of livers of fishes are for the most part the same as those found in the livers of their mammalian counterparts, there are some differences. Exocrine pancreatic tissue is found in the connective tissue of the hepatic portal vein (variable with species) and some of its branches within the livers of certain species (e.g., carp) (Figueiredo-Fernandes et al., 2007; Vogt and Segner, 1997). This is not the general case in mammalian species. The Kupffer cell, a resident macrophage in hepatic sinusoids of mammals, is not found in most fish species (see Table 7.1). For those species in which the cells are present, their functions have been only partially determined (Hampton et al., 1987). Although the other cell types (see descriptions above) are found, their role in hepatotoxicity in fish has not been well characterized, and we have little or no information regarding signaling between various resident cells. This area requires future attention. The role of these cells in immune reactions and in initiating and mediating liver toxicity is not known. We do not know why certain hepatoxicants preferentially damage one cell type and if there exists preferential uptake or metabolism of certain toxicants by specific liver cell types. How the cells of the hepatic sinusoids and those involved in immune function influence liver pathogenesis is an area that so far has not been addressed in fishes.

Metabolism as Factor in Liver Toxicity

Xenobiotic metabolism and activation is an important factor in liver toxicity. Toxicity of a chemical may be due to the parent compound or to a metabolite generated by metabolic conversion of the former to reactive electrophiles that combine covalently with critical cellular macromolecules such as proteins, DNA, or RNA. The liver is the major site of biotransformation in fish and performs both phase I and phase II reactions of xenobiotic metabolism. Liver xenobiotic metabolism has important consequences

for both systemic and organ-specific toxicity. First, hepatic biotransformation directly relates to bioaccumulation of lipophilic substances in fish (Schultz and Hayton, 1999; Sijm et al., 1993; Stuthridge et al., 1997). Second, hepatic metabolism influences the toxicity of xenobiotics either through effects on tissue doses of the toxicant or through generation of metabolites with either reduced or enhanced toxicity (detoxification/toxification) of the parent compound (Debruijn et al., 1993; Keizer et al., 1991; Lech and Bend, 1980). Hepatic metabolism is also important in determining species differences in the bioaccumulation (Schultz and Hayton, 1999) and toxicity (Hasspieler et al., 1994; Wirgin and Waldman, 2004) of xenobiotics in fish.

For mammals, numerous studies have shown the relation between the tissue-specific formation of a reactive intermediate and hepatotoxicity. Further differences in the distribution of xenobiotic-metabolizing enzymes among the particular liver cell types predispose them to xenobiotic toxicity. In fish a number of studies have analyzed liver conversion of xenobiotics in fish; however, information relating hepatic biotransformation to hepatotoxicity is often lacking. The distribution of metabolic enzymes such as CYP1A in the different cell types of fish liver and their roles in subsequent development of liver toxic lesions have received little attention, with the one exception being liver carcinogenesis (see chronic biliary toxicity discussion in this chapter).

One group of environmental contaminants that fish are often exposed to and for which hepatic metabolism is an important toxicokinetic and toxicodynamic determinant are polycyclic aromatic hydrocarbons (PAHs) (Altenburger et al., 2003). Several studies have investigated the spectrum of metabolites produced by fish liver preparations and found a high proportion of 7,8- and 9,10-dihydrodiols (Lemke and Kennedy, 1997; Morrison et al., 1985; Nishimoto et al., 1992). Induction of metabolic enzymes, however, can result in a shift of the metabolite spectrum (van Schanke et al., 2000). Information is available from both field and laboratory studies on the association of exposure to PAHs, enhanced expression of hepatic biotransformation enzymes, and the occurrence of DNA adducts and toxicopathic liver lesions, including neoplastic alterations (Aas et al., 2001; Dey et al., 1993; James et al., 1988; Lesage et al., 2001; Malins et al., 1988; Myers et al., 1987, 1994, 1998; Orsler et al., 1999; Palmeira et al., 2003; Spitsbergen et al., 2000a,b; Vogelbein et al., 1999). In a field study on the Puget Sound, Horness et al. (1998) were able to establish statistically significant threshold levels for the association of PAH concentrations in the sediments and the occurrence of neoplastic liver alterations in flatfish, this providing strong evidence for a causative role of hepatic PAH metabolism in carcinogenesis of the fish species.

Another example where hepatic metabolism of xenobiotics is an important determinant in toxicity is related to the hepatocarcinogenic mycotoxin aflatoxin B_1. In the liver of trout, aflatoxin B_1 is metabolized to aflatoxicol B_1 and excreted as aflatoxicol B_1 glucuronide (Loveland et al., 1984). An increase in metabolism of aflatoxin B_1 and enhanced biliary excretion as glucuronide following pretreatment by the CYP1A inducers β-naphthoflavone or Arochlor® 1254 significantly reduced liver DNA adduct formation (Bailey et al., 1987). Further, pretreatment of trout with indole-3-carbonyl (an inducer of glutathione *S*-transferase) also reduced the carcinogenicity of aflatoxin B_1 (Goeger et al., 1986). Differences in aflatoxin metabolism are also likely to be responsible for the known differences in aflatoxin sensitivity between rainbow trout and Coho salmon (Bailey et al., 1984; Coulombe et al., 1984). A contradictory example appears to be the zebrafish, as adult zebrafish rapidly metabolize and excrete aflatoxin B_1. Both *in vitro* and *in vivo* studies indicate that this species has the capacity to bioactivate aflatoxin B_1 to its reactive intermediate, and the level of hepatic DNA adducts is only moderately lower than observed in trout (Troxel et al., 1997). An apparent contradiction to these findings is the reported resistance of zebrafish when aflatoxin B_1 was administered in the diet. It is possible that mechanisms other than biotransformation protect the zebrafish against aflatoxin toxicity.

A further example where hepatic metabolism is at least partially responsible for chemical toxicity is the procarcinogen 2-acetylaminofluorene (AAF), which induces liver tumors in fish at a much lower rate than in rodents (James et al., 1994). This apparently higher resistance of fish to the hepatocarcinogenicity of AAF seems to be related to AAF metabolism. For medaka, guppy, and trout, major metabolites of AAF generated in the liver are hydroxy-AAFs, while the carcinogenic metabolite *N*-hydroxy-AAF is formed at only low rates (James et al., 1994; Steward et al., 1994). Thus, the metabolic differences between fish and mammals may partially explain the resistance of fish and susceptibility of mammals to AAF-induced hepatocarcinogenesis.

Responses of Fish Liver to Reference Hepatotoxicants

During the early stages of development of new animal models for toxicological research, there is a validation phase that demonstrates that the animal responds to compounds of proven toxicity (in this case, reference hepatotoxicants) and that the response has features similar to those seen in established models (usually rodents). Studies investigating three reference hepatotoxicants—acetaminophen (AP), allyl formate (AF), and carbon tetrachloride (CCl_4)—were reviewed by Hinton et al. (2001). The interested reader is referred to that review, as we provide only a brief summary here.

Summary of Acetaminophen Review

Although AP produces centrolobular necrosis in rodent liver, exposed rainbow trout responded with focal rather than zonal necrosis and the extent of necrosis was less. Trout exposed to AP responded with an increase in serum glutamic pyruvic transaminase (SGPT) in a dose-dependent manner (Droy, 1988) but one that was at least an order of magnitude lower than reported for hamsters and rats. Other parameters of hepatic toxicity failed to show a response relationship in AP-exposed trout. Responses of mullet (*Mugil cephalus*) were similar (Thomas and Wofford, 1984); for example, exposure to AP failed to cause significant depletion of glutathione stores, and the authors concluded that mullet liver failed to produce a sufficiently toxic metabolite. Because fish have the capacity to form certain reactive intermediates of AP, differences in metabolism were not regarded as the sole explanation for the reduced levels and the different patterns of hepatotoxicity observed. AP deserves further analysis in fish liver toxicity. It is an example of a reference hepatotoxicant that produces discrete but lowered responses, the pattern and characteristics of which will likely lead to improved understanding of fish liver toxicity in general.

Summary of Allyl Formate Review

Allyl formate is the only reference hepatotoxicant studied in fish that has selective periportal zonal toxicity in rodent liver. In rainbow trout, the magnitude of toxic response following AF exposure was appreciable, with morphologic, biochemical, and functional changes elicited (Droy et al., 1989). Ultrastructural analysis of AF-induced hepatotoxity showed that endothelial cells of sinusoids were targeted and that the hemorrhagic necrosis likely involved early lethal toxicity to this cell type. When the concentration was lowered but the route of administration maintained, enhanced morphologic response in perivenous areas suggested a zonal response. AF hepatotoxicity is likely mediated via alcohol dehydrogenase, but histochemical studies of trout liver indicated no preferential localization of this enzyme (Schar et al., 1985). Similarly, glutathione localization in trout liver is homogeneous and likely would not lead to a heterogeneous response to AF. AF response in trout was appreciable, and further analysis of its time course over a broader range of lowered concentrations will likely prove fruitful in determining the role of signaling between endothelial cells and hepatocytes in fish liver toxicity.

Summary of Carbon Tetrachloride Review

The classic example of biotransformation-mediated generation of toxicity in mammalian liver is the cytochrome P450-dependent conversion of CCl_4 and chloroform to free radicals such as $\cdot CCl_3$ and $CCl_3OO\cdot$, which then initiate lipid peroxidation, acute liver injury (e.g., necrosis), and, under chronic conditions, liver neoplastic changes (Treinen-Moslen, 2001). In rodents, CCl_4 is metabolized to CCl_3, which rapidly combines with oxygen to form the very reactive CCL_3O_2 (Packer et al., 1978); therefore, different free-radical species of CCl_4 are present under aerobic and anaerobic conditions in rat liver. Hepatic injury is maximal in centrolobular areas of mammalian liver where oxygen tension is lowest (Burk et al., 1984a,b). Also, hypoxia potentiates covalent binding of metabolites to rat liver microsomal lipids and proteins (Shen et al., 1982).

Fish appear to be more resistant to the toxic action of CCl_4. In the study by Droy et al. (1988), acute oral dosing of fish with CCl_4 was selected because this method of delivery was related to a more natural

exposure route and obviated the solvent effect seen after intraperitoneal injections. Necrotic hepatocytes were more abundant in the high-dose group (3 mL CCl_4 per kg), and a loss of cytoplasmic basophilia with swelling characterized the affected hepatocytes. In rats used as biological controls for the experiment, the researchers found a quantity of necrosis not approximated by trout even after greater doses of the toxicant. Also, with respect to the chronic toxicity of CCL_4, trout appear to be less sensitive. In rainbow trout, chloroform enhanced the hepatocarcinogenicity of aflatoxins (Kotsanis and Metcalfe, 1991), while in medaka chronic exposure to chloroform was not hepatocarcinogenic (Toussaint et al., 2001a). The apparent higher resistance of fish to CCl_4-induced injury might be because fish liver lacks the metabolic machinery capable of bioactivating CCl_4. Evidence against this includes the report of increased lipid peroxidation and ^{14}C-labeled CCl_4 residues in trout liver 6 hours following exposure to a single intraperitoneal injection of 1 mL CCl_4 per kg (Statham et al., 1978). Also, in mullet (*Mugil cephalus*) and croaker (*Micropogonias undulates*), increased lipid peroxidation occurred as a result of CCl_4 treatment (Wofford and Thomas, 1988). Further, *in vitro* studies have provided strong evidence that fish liver cells metabolize CCl_4 to free radicals (Rabergh and Lipsky, 1997); also, Toussaint et al. (2001a) found that chloroform is metabolized in medaka liver.

A second hypothesis that could explain why CCl_4 is less hepatotoxic in trout would be the presence of higher amounts of antioxidants or free-radical scavengers such as glutathione. When trout and rat were compared after exposure to monochlorobenzene (Dalich and Larson, 1985), rat hepatic glutathione was about three times that of trout; therefore, it seems unlikely that the lack of susceptibility of trout to CCl_4 can be explained by excessive amounts of antioxidant. In addition, Toussaint et al. (2001b) showed that CCl_4 treatment of trout hepatocytes resulted in a serious depletion of cellular glutathione levels.

Droy (1988) considered the possibility that interspecies differences in oxygen tension and glutathione distribution patterns might explain the trout response to CCl_4. Glutathione is effective against CCl_4-induced lipid peroxidation in rat liver but only in the presence of oxygen, when CCL_3O_2 is readily formed (Burk et al., 1983). The relative abundance of glutathione in portal vs. centrolobular areas also contributes to the protection seen in the portal hepatocytes of rodent liver (Smith et al., 1979); therefore, glutathione appears to protect rat liver portal hepatocytes from CCl_4 because of the preferential distribution of glutathione and the likely ability of this compound to scavenge CCL_3O_2 but not CCl_3. Droy (1988) reported a refractiveness in trout to the hepatotoxic effects of CCl_4, with scattered individual hepatocyte necrosis being the typical response. Perhaps the inability of CCl_4 to produce appreciable toxicity in trout liver could be due to comparatively high oxygen tension. Under these conditions, CCl_3 would be converted to CCL_3O_2, which would then combine with glutathione and be rendered inert.

Exposure of rodents to CCL_4 produces a mosaic of altered hepatocytes in centrolobular zones and an apparent sparing of hepatocytes in the periportal zone, the more oxygenated zone. Zonal reactions are not seen following exposure of fish to CCl_4. When carrier-solvent-associated changes are removed from consideration, CCl_4 is not a particularly strong hepatotoxicant in fishes. Scattered individual hepatocyte necrosis is the usual response. Differences in CCL_4 metabolism, levels of glutathione, metabolic attack on the parent compound, oxygen tension, and absence of the Kupffer cell in trout have been suggested as possible reasons underlying this differential response.

Taken together, this body of work indicates that livers of fishes respond to reference hepatotoxicants but that the response is quantitatively different from that of rodents. The pattern of morphological alteration is different, likely reflecting innate differences in hepatic architecture and quantitative as well as qualitative differences in metabolism of parent compounds. Additional effort is needed to better characterize hepatotoxicity in fishes. Increased understanding of structure (especially architecture), of the roles of hepatocytes and nonhepatocytic cell types in toxic responses, and of metabolism will enhance our ability to interpret alterations following exposure to drugs, personal-care products, pollutants, and biotoxins.

As we shall see in the subsequent section, elegant and detailed ultrastructural studies in livers, isolated primary cells, and fish-derived cell lines have made it possible for us to demonstrate hepatocellular adaptation following exposure to reference hepatotoxicants or develop an appreciable assemblage of individual environmentally relevant compounds as is suggested by studies from wild fish collected at heavily contaminated sites.

Hepatocytes Under Toxic Exposure

Changes in hepatocytes do not necessarily signify overt toxicity and may represent sublethal cellular change as seen during adaptation to toxicants or due to an altered equilibrium state in injured tissues or cells. A detailed consideration of ultrastructural hepatic changes has been provided recently by Braunbeck (1998) and Au et al. (2004), so this review is brief. The examples are arranged by inclusion body and by specific organelle.

Lipopigments

Lipofuscin and ceroid share very similar morphology and physicochemical properties. Lipofuscin is regarded as the classical age or stress pigment, and the accumulation of ceroid is indicative of cell pathology. Lipopigments are widely regarded as the end products of lipid peroxidation, which is a free-radical chain reaction. Such a reaction is initiated when some unidentified free radical abstracts a methylene H-atom from the unsaturated fatty acid and then reacts with oxygen to yield a hydroperoxy radical. Termination of the chain reaction is often associated with polymer formation, and copolymerization of oxidized lipids with protein and other compounds would form lipopigments (Dayan and Wolman, 1993; Donato, 1981; Wolke et al., 1985). Lipopigments tend to accumulate in the lysosomal compartment as residual bodies (Holtzman, 1976; Park and Chi, 1992; Yin, 1996). This is exemplified by the fact that secondary lysosomes have often been found in the vicinity of lipopigments in hepatocytes of fishes. Early ultrastructural studies of Elbe flounder and dab collected from sites highly contaminated with organochlorines and heavy metals showed an unusual extent of transformation of lysosomes into lipofuscins and were in close association with the large lipid droplets in hepatocytes (Köhler, 1990; Köhler et al., 1992). Likewise, livers of mummichog inhabiting a PAH-contaminated (creosote) environment often exhibited sublethal cellular damage, including the accumulation of ceriod and lipofuscin in non-neoplastic hepatocytes bordering carcinomas (Vogelbein, 1993). Increased lipofuscin was also reported in the liver of juvenile grey mullets after exposure to the algicide atrazine (Biagianti-Risbourg and Bastide, 1995). A recent survey in the San Diego Bay in California (Exponent, 2003) reported that the prevalence of lipofuscin in hepatocytes of the spotted sand bass (*Paralabrax maculatofasciatus*) was most strongly associated with shipyard sites that were characterized by high levels of organic contaminants (e.g., PCBs, PAHs) in sediments. It is important to be able to quantify hepatic lipopigment accumulation with levels of pollutants. Stereological analysis was employed to quantify cytological changes in the livers of immature sole (*Solea ovata*) and juvenile orange-spotted grouper (*Epinephelus coioides*) exposed to benzo(*a*)pyrene (BaP). A dose–response relationship was generally demonstrated between volume density, absolute volume, numerical density, and absolute number of lipopigment accumulation and levels of BaP under both laboratory and field conditions, regardless of the route of PAH uptake (Au, 2004; Au and Wu, 2001; Au et al., 1999). The accumulation of lipopigment in mammals (ceroid-lipofuscinosis) is a pathological symptom known as Batten's disease. The resulting deleterious effects include blindness, seizures, dementia, and premature death (Martinus et al., 1991; Palmer et al., 1986; Yin 1996). Furthermore, large amounts of undegradable lipofuscin and ceroid in human fibroblasts were shown to increase the susceptibility of cells to oxidative stress, disturbance of lysosomal function, and cell death (Terman et al., 1999a,b). Condensation of hepatocellular content was often observed in the vicinity of lipopigments in *Solea ovata* exposed to BaP (10 mg/kg BaP), which may suggest severe lipid peroxidation in hepatocytes (Au et al., 1999). It is therefore clear that intracellular lipopigment accumulation is indicative of adverse cytological effects. Conceivably, an excessive amount of lipopigment interferes with vital cellular functions and decreases the survival of affected cells, which may eventually decrease the fitness of fish in the contaminated environment.

Lysosomes

These membrane-delimited organelles contain hydrolytic enzymes for breaking down substances within a cell (autophagy) or substances that have been taken in from outside the cell (heterophagy)

(Bozzola and Russell, 1992; Holtzman, 1976). Alterations to lysosomal structure are usually associated with a reduction in the stability of lysosomal membranes and an increase in lysosomal enzyme activities, which may pose a potential risk to the health of the cell or the individual organism (Köhler, 1991; Moore, 1993). Reduced lysosome membrane stability and augmentation of lysosomes have been reported in the livers of feral fish collected from highly contaminated waters, and in certain cases, including exposure to heavy metals, increases in lysosome number were also observed (Baker, 1969; Braunbeck, 1998; Köhler, 1991; Köhler et al., 1992, 2002; Lanno et al., 1987; Lowe et al., 1992; Segner and Braunbeck, 1990b; Woodworth et al., 1998). A recent study by Köhler and colleagues (Köhler et al., 2002; Wahli, 2002) demonstrated a good dose–response relationship between the severity of lysosomal alterations in livers of European flounder and the levels of xenobiotic pollution. Moreover, increase in the size of lysosomes with accumulation of lipid and electron-dense content (lipopigment) coincided with decreased lysosome membrane stability in fish livers (Köhler et al., 2002). More prominent transitions of primary to tertiary lysosomes or lipopigments were observed in the livers of *Solea ovata* and *Epinephelus coioides* subjected to BaP or PAH exposure. In general, lysosome alterations are indicative of early cytopathological responses; they are sensitive but nonspecific to a wide range of contaminants, including a diverse range of toxic metals and organic chemicals (e.g., PAHs, PCBs, oil-derived hydrocarbons).

Peroxisomes

These single-membrane-limited organelles are ubiquitous in eukaryotic cells, where they play key roles in major metabolic functions such as lipid metabolism (e.g., fatty acid β-oxidation) and cellular respiration involving the metabolism of H_2O_2 (e.g., production of oxidases and catalase) (Fahimi et al., 1983). Massive proliferation of peroxisomes may occur in animals after treatment with certain xenobiotics and drugs (Fahimi and Cajaraville, 1995; Gibson and Lake, 1993; Moody, 1994). Peroxisome proliferation is defined as an increase in either peroxisomal volume or numbers which may not always be accompanied by an induction of peroxisomal enzymes (Reddy and Mannaerts, 1994). Catalase activity is one of the marker enzymes used for assessing peroxisome proliferation biochemically (Moody, 1994). Channel catfish (*Ictalurus punctatus*) and dab (*Limanda limanda*) exhibited an increase in catalase and superoxide dismutase activity after exposure to PAH-contaminated sediments (Di Giulio et al., 1993; Livingstone et al., 1993). Significant increases of volume density and absolute number of peroxisomes were demonstrated in immature sole (*Solea ovata*) after intraperitoneal injection of fish with BaP (Au et al., 1999). Induction of peroxisome proliferation has been reported in various animal tissues in response to PAHs and other xenobiotics (Bentley et al., 1993; Fahimi and Cajaraville, 1995; Krishnakumar et al., 1995, 1997; Reddy et al., 1987). Inconsistent peroxisome reactions were found in the hepatocytes of rainbow trout and zebrafish in response to various classes of toxicants; for example, the number of peroxisomes was reduced in response to 4-chloroaniline and lindane, but proliferation occurred in response to 3,4-dichloroaniline (Braunbeck, 1990; Braunbeck and Appelbaum, 1998). Species-specific variation and toxicant specificity are the factors to be considered when studying the responses of peroxisome proliferation in fish. It is also known that peroxisomal enzyme activity in fish could also be affected by other biotic factors, such as sex, and abiotic factors, such as salinity, temperature, season, and feeding habit (Braunbeck et al., 1987; Palace et al., 1993; Radi et al., 1987; Rocha et al., 1999).

Rough and Smooth Endoplasmic Reticulum

The hepatic cytochrome P450–monoxygenase system is a coupled electron transport system situated in the endoplasmic reticulum of hepatocytes (Mansuy, 1998). Early proliferation of the hepatic ER system was reported for fish exposed to PAHs, such as 3-methylcholanthrene (3-MC) and PCBs (Klaunig et al., 1979; Schoor and Couch, 1979). BaP stimulated rough and smooth ER proliferation in the livers of *Solea ovata* and *Epinephelus coioides* regardless of the route of exposure (water vs. intraperitoneal) (Au, 2004; Au and Wu, 2001; Au et al., 1999; Lemaire et al., 1992). A wide range of xenobiotics has been shown to induce hepatic ER proliferation (Braunbeck, 1998). In general, fish hepatic ER induction is nonspecific but sensitive to environmental contaminants.

Nucleus and Mitochondria

The formation of binucleated hepatocytes was observed in livers of *Epinephelus coioides* at one week after oral exposure to BaP (12.5 μg/g BaP) and the frequency of occurrence increased with the exposure time. A dose–response relationship was demonstrated between the numerical density of mitochondria and BaP exposure (up to 5 mg/kg BaP) in *Solea ovata* (Au et al., 1999, 2004); the higher abundance of mitochondria after BaP treatments was thought to indicate active cellular metabolism to meet an increased high demand for ATP. The depletion of glycogen content was observed in a majority of hepatocytes upon exposure to BaP, regardless of the fish species and the route of exposure (Au et al., 1999, 2004). Glycogen depletion is a nonspecific adaptive response of hepatocytes to a large variety of xenobiotics and is generally regarded as a parameter for indicating acute or short-term stress (Arnold et al., 1995; Biagianti-Risbourg and Bastide, 1995; Braunbeck and Volk, 1991; Eurell and Haensly, 1981; Gluth and Hanke, 1985; Rojik et al., 1983). Lipid storage in fish liver can be both decreased and increased under toxicant exposure. Oral administration of 0.5 μg endosulfan per kg food to juvenile carp reduced the volumetric content of hepatic lipid from 14% to 5%, which may represent a general stress response as individual hepatocyte volume was also reduced by 50% (Braunbeck and Appelbaum, 1998). In both male and female zebrafish, exposure to lindane induced a pronounced steatosis microvesicular fatty transformation of the rough ER cisternae (Braunbeck et al., 1990a). Induction of steatosis by toxicants has been also reported from mammalian liver, and the underlying mechanism may be the blockage of lipoprotein secretion from the rough ER and Golgi system (Baglio and Farber, 1965).

Liver Alterations in Fish Exposed to Environmental Pollutants

The relationships between histopathologic lesions in fish livers and exposure to persistent environmental contaminants were thoroughly studied in large-scale surveys conducted by the U.S. National Marine Fisheries Services (NMFS). A total of 18 marine bottom fish species collected from 45 sites on the Pacific coast (Malins et al., 1988; Myers et al., 1993; Varanasi et al., 1989) and 22 sites on the Northeast coast (Johnson et al., 1992a,b) were included in the surveys. Results demonstrated that megalocytic hepatosis (MH), foci of cellular alteration (FCA), and biliary and hepatocellular neoplasms were prevalent in the English sole and other bottom fish species (e.g., rock sole, starry flounder, Pacific staghorn sculpin, Dover sole, and white croaker), and their prevalences were closely associated with exposure to PAHs, PCBs, and DDT in sediment, stomach content, and liver (Myers and Rhodes, 1988; Myers et al., 1993). Similar extensive surveys have also been carried out in Canada, Europe, and Australia to assess the impact of environmental contaminants on fish health (Au, 2004).

These survey data generally show a good correlation between the concentrations of various persistent chlorinated hydrocarbons and metals and liver lesions (Köhler, 1990; Malins et al., 1988; Myers et al., 1992). A recent study by Stehr et al. (2004) in Vancouver Harbour in Canada also demonstrated that the occurrence of toxicopathic liver lesions in English sole was statistically associated with concentrations of aromatic hydrocarbon (AH) metabolites in the sediment and AH metabolite levels measured in bile. Also, in numerous studies on freshwater environments, good correlations between water quality and liver histopathology have been demonstrated (Handy et al., 2002; Schmidt-Posthaus et al., 2001; Schwaiger et al., 1997; Teh 1997). Evidently, fish livers are highly sensitive to pollutant exposure. Fish liver histopathology is indicative of the general health condition of fish and levels of toxic xenobiotics, carcinogens, and urban pollution.

Other histopathologic responses, such as escalated macrophage immigration and subsequent increases in the number of macrophage aggregates (MAs), were observed in the livers of English sole and winter flounder with liver neoplasms (Myers et al., 1987) as well as in the livers of fish inhabiting degraded environments (Murchelano and Wolke, 1991). The use of hepatic MA hyperplasia was recommended as an indicator of health of grey mullet juveniles in contaminated coastal environments (Biagianti-Risbourg, 1993). Fish exposed to pulp mill effluent also showed considerably greater MA in the spleen of winter flounder (Khan and Payne, 1997) and in the livers of common jollytail and Tasmanian blennies (Woodworth et al., 1998). Moreover, field studies have been supported by controlled laboratory investigations,

which have demonstrated early cytological changes in fish liver and damage from exposure to a wide range of pollutants, including PCBs (Klaunig et al., 1979; Köhler, 1989), 3-methylcholanthrene (Schoor and Couch, 1979) diethylnitrosamine (Couch, 1993, Hinton et al., 1988), 4-nitrophenol and 4-chloroaniline (Braunbeck et al., 1989, 1990a), BaP (Lemaire et al., 1992), and linuron (Oulmi et al., 1995). With the recent advent of digitized imaging for electron microscopy and computer software for stereological analysis, quantification of cytological changes on two-dimensional ultrathin sections is no longer a time-consuming process. Results of morphometry provide more useful quantitative data on cytological changes in response to contaminants.

Certain persistent environmental pollutants (e.g., PAHs, aromatic amines, nitroso-compounds, azo compounds) were shown to be potent hepatocarcinogens in fish (Moore and Myers, 1994). Hepatic carcinogenesis is particularly useful to indicate chronic toxicity in fish. Other hepatic lesions, such as FCA, MH, and hepatocellular nuclear pleomorphism (NP), were considered as early pathological stages in the formation of liver neoplasms (Hinton and Lauren, 1990; Hinton et al., 1992, 2001; Myers et al., 1987; Simpson and Hutchinson 1992); however, the etiology of preneoplastic liver lesions in relation to pollutant exposure remains largely unknown. The synergistic and antagonistic interactions of chemicals in water and sediment make it difficult to study cause-and-effect relationships. Moreover, it should be noted that liver tumors and histopathology are manifestations of chronic toxicity associated with prolonged latency periods. These lesions have great overall significance, especially when prevalences are used to suggest an epizootic at a localized site; however, their presence does not tell us about recent alterations in environmental quality. For many fish, migration is an annual event that makes it difficult to assess temporal and spatial aspects of exposure. As we shall see below, the use of biochemical and cytological endpoints may have more to offer especially when applied to younger fish for which habitats are known.

The correlation of biochemical and morphological (ultrastructural) changes is important. Not only do organelles and inclusion bodies show changes in hepatocytes of organisms residing at contaminated sites or exposed to usually single pollutants in controlled laboratory studies, but also a correlation exists between these alterations and certain biochemical measures (Grinwis et al., 2000).

Although most of the earlier biomarker studies concentrated on linking individual biochemical and morphological responses to exposure and effects of pollutants, only a few studies related biochemical endpoints (e.g., hepatic EROD/MFO activities) to quantitative ultrastructural changes in livers of fish upon exposure to toxicants (Chui et al., 1985; Hugla and Thome, 1999; Klaunig et al., 1979; Kontir et al., 1984; Schoor and Couch, 1979). If hepatic EROD activity can be related to quantitative hepatocytological damages, then the measurement of EROD activity would then indicate not only exposure but also adverse biological effects. Furthermore, the use of hepatic EROD as a biomarker would be more useful if linked to important biological processes. Some of the associated hepatocytological changes in fish may also serve as potential effect biomarkers for the early detection of exposure to environmental pollutants.

Recent studies sought to establish the relationship between quantitative hepatocytological changes and EROD activities in *Solea ovata* and *Epinephelus areolatus* exposed to PAHs and to provide important information regarding the use of such a relationship. Immature individuals of the demersal fish *S. ovata* were exposed intraperitoneally to benzo(*a*)pyrene, and quantitative cytological alterations were quantified (Au et al., 1999). A dose–response relationship was shown between exposure to BaP and changes in hepatic EROD activities. A Spearman rank correlation analysis revealed correlation between EROD activities and number or absolute volume of peroxisomes as well as lipofuscin granules in hepatocytes.

In a subsequent field study, chemical analysis of sediment from a dump site showed high levels of PAHs and PCBs (Au and Wu, 2001). Sexually immature fish from this site exhibited significantly higher EROD activity compared with counterparts from a reference site. In this case, a significant correlation was found only between EROD activity and volume density (Vv) of hepatic lipopigments. When this correlation was tested in juveniles of another species, the areolated grouper (*Epinephelus areolatus*), it was shown to exist (Au et al., 2004). These findings seem reasonable given the fact that lipopigment is a product of lipid peroxidation and could signify oxidative stress in cells. Excessive intracellular lipopigment accumulation could interfere with vital cellular functions and decrease survival of affected cells

(Terman et al., 1999b). Intracellular lipopigment accumulation, indicative of adverse cytological effects, means that the biomarkers could be signifying exposure and adverse effect. Moreover, both the cytological and biochemical changes proved reversible, subsiding upon withdrawal of BaP exposure (Au et al., 2004). Hepatic EROD activity and lipopigments may therefore be useful to indicate recent exposure of the fish to BaP and PAHs.

Proliferation of the smooth and rough ER systems was repeatedly demonstrated in the hepatocytes of fish exposed to xenobiotic compounds (Au et al., 1999, 2004; Braunbeck and Appelbaum, 1998; Lemaire et al., 1992). A dose–response relationship was not obvious between ER proliferation and levels of BaP (Au et al.,1999). Earlier correlation studies demonstrated inconsistent relationship between biochemical and hepatic ER changes in response to toxicants. Catfish exposed to the PAH 3-methylcholanthrene (3-MC) (Schoor and Couch 1979) and barbell and mullet exposed to PCBs (Hugla and Thome, 1999; Klaunig et al., 1979) showed good correlation between proliferation of ER system with an increase in EROD/MFO activities. In contrast, subsequent studies on rainbow trout exposed to 3-MC (Kontir et al., 1984) and speckled trout exposed to chlorinated diphenyl ethers (Chui et al., 1985) showed no corresponding changes in ER with increased EROD/MFO activities.

Linkage of Biological Responses to Environmental Pollutants in Water and Sediment

Over the past 15 years, numerous field and laboratory studies have been conducted from all over the world to link biological responses (i.e., molecular, biochemical, histopathological, immunological, physiological) in fish to environmental pollutants (ANZECC, 1992; Au, 2004; Payne et al., 2003; Stagg et al., 1998), with an attempt to estimate exposure to and effects of environmental contaminants. A variety of biological responses, at different levels of organization, are candidate biomarkers or bioindicators for pollution monitoring. According to Peakall (1992), biomarkers are measures of suborganismal responses (i.e., molecular, biochemical, physiological, or histological response) in organisms that can demonstrate exposure to or the effects of environmental contaminants. In general, molecular responses will first occur when an organism is subject to stress. This is followed by a variety of homeostatic mechanisms in biological systems that enable the organism to restore to original conditions or to an altered state of equilibrium that is consistent with life. Biological responses at a higher biological organization (i.e., population, community, and ecosystem responses) occur if these mechanisms fail to restore to normal conditions.

Many of the molecular biomarkers used in environmental monitoring are measured from liver tissue of fish. One example is the CYP1A biomarker. The induction of the fish liver cytochrome P450 system, especially the enzyme 7-ethoxyresorufin-*O*-deethylase (EROD), has been commonly used as a biochemical biomarker for exposure to PAHs, PCBs, and petroleum hydrocarbons (Ahokas et al., 1976; Au, 2004; Au and Wu, 2001; Au et al., 1999; Behrens and Segner, 2005; Beyer et al., 1994; Bucheli and Font 1995; Collier et al., 1995; Holdway et al., 1995; Livingstone et al., 1997; Wu et al., 2003). CYP1A/EROD induction is sensitive to low levels of contamination exposure and could be more sensitive than direct measures of contaminant residuals (Collier et al., 1995); however, dose–response studies reveale CYP1A induction reaches maximal levels after exposure to moderate levels of contaminants (Banka et al., 1997; Fent et al., 1998; Wu et al., 2003). In addition, response time for EROD to PAHs appeared to be highly variable; for example, hepatic EROD activities in the *Solea ovata* subsided to background levels 7 days after an intraperitoneal injection of BaP (Au et al., 1999), while EROD activities in the dietary BaP-treated grouper fish subsided at week 4 of exposure and throughout the depuration period (Au, 2004). In contrast, sustained EROD activity was found in rainbow trout fed with 2,3,4,7,8-pentachlorodibenzofuran during the 31-day exposure period as well as the 180-day depuration period (Muir et al., 1990). The difference in response time was probably attributable to different half-lives of these compounds or the route of exposure in fish. Generally, induction of hepatic EROD activity is sensitive to a wide range of xenobiotics; the increase tends to be transient and subsides shortly after exposure is terminated. Importantly, a good relationship has been established between hepatic EROD induction and lipopigment accumulation, a harmful biological effect. The ecological relevance and

TABLE 7.7

Summary of Application of Fish Liver Histopathology in Major Pollution Monitoring Programs

Pathologic Entity	Monitoring Programs	Refs.
Liver tumor	Recommended for use by the ICES	ICES (1989, 1996, 1997)
Hepatic lesions	Multidisciplinary surveys by the U.S. National Marine Service	WGBEC (2002)
	Fisheries services on the Pacific and Northeast coasts	Malins et al. (1988)
	Gulf of Mexico integrated monitoring programs	Varanasi et al. (1989); Johnson et al. (1992a,b); Wolfe (1992); Myers et al. (1993); USEPA (2000)
Macrophage aggregate	NOAA marine monitoring programs and U.S. National Estuary Program in Virginia estuaries	Wolf et al. (1992); Strobel et al. (1999)
Lycrosomal integrity	North Sea pollution monitoring in Germany	Broeg et al. (1999)

ecotoxicological value of the EROD biomarker are significant (Au, 2004). This biochemical biomarker has proved successful in identifying regions impacted by industrial pulp and paper effluents (Munkittrick et al., 1991). The use of hepatic EROD induction in fish can be found in regulations in Canada and Australia (Collier et al., 1992, 1995; Holdway et al., 1994, 1995), and such an approach is considered potentially suitable for long-term monitoring of the marine environment in Hong Kong (EPD, 2003).

Histocytopathological alterations of fish liver are frequently used in monitoring programs as markers of fish health. Numerous field studies have reported liver histopathological changes in fish from contaminated environments, and liver histopathology has been found to be a sensitive indicator of pollution stress and impaired fish health (Handy et al., 2002; Schwaiger, 2001; Teh, 1997; Vethaak and Jol, 1996; Zimmerli et al., 2007). A drawback of liver histopathology to assess toxicant stress can be the qualitative nature of histological data; however, this can be overcome by using standardized, semiquantitative evaluation schemes (Bernet et al., 1999).

It is likely that the histocytopathological symptoms described in fish liver may decrease the fitness of the individual through disturbed metabolic homeostasis and proper functioning of vital biological processes (e.g., detoxification). Fish liver pathological symptoms are of significant ecological relevance. Moreover, hepatic lesions are highly sensitive to pollutant exposure, and certain lesions have been well correlated with contaminant exposure (Au, 2004). Some histopathological incidences are species specific; the restrictive natural distributions of many species may make monitoring results not directly comparable between various locations. Monitoring the presence of liver tumors in young fish may not be suitable due to a long latent period for disease development. Except for liver tumors that have a well-defined and validated morphology (Boorman et al., 1997), the technical difficulty is high for symptom diagnosis. Moreover, certain hepatic lesions, such as macrophage aggregates, relate to a large number of possible confounding factors (e.g., season, reproductive stage, migration pattern, nutritional conditions). This makes it necessary to strictly standardize sampling protocols, which sometimes may be difficult. This problem is further exacerbated by the low incidence of MAs in normal fish livers, and a large sample size is necessary to discern statistically significant differences between fish population in different sites or times. In general, the hepatic lesions reviewed are responsive to a variety of pollutants and are therefore indicative of the general quality of the environment rather than specific types of pollutants. Some histopathological symptoms of fish livers have already been applied in field monitoring (Table 7.7). Liver pathologies are currently classified as Category II by the OSPAR Commission, which includes criteria for quality assurance procedures that are not yet in place although they may be used for monitoring (WGBEC, 2002).

In contrast, cytological structures (e.g., lipopigments and lysosomes) that occur commonly in most living cells are easy to identify. The cause-and-effect relationships and detailed mechanisms leading to the development of most pathological symptoms are becoming clear. Fish hepatic lipopigment has shown promise as a cytological marker of effects of PAHs and BaP (Au, 2004; Au and Wu, 2001; Au et al., 1999). Lipopigment content was chosen as one of the battery of biomarkers to assess long-term effects

of oil pollution on mussels and fish in the Ligurian Sea in Italy (Viarengo et al., 2001). Identification of lipopigment *per se* is not difficult, but quantification of abundance and size may not be cost effective for routine monitoring. Biochemical measurement of lipid peroxidation could be employed to indicate formation of lipopigments in liver.

Lysosomal alterations in fish hepatocytes have been recommended as potential cytological biomarkers for environmental pollutants. Köhler et al. (2002) demonstrated a good dose–response relationship between the severity of lysosome alterations in liver of *Platichthys flesus* and the levels of xenobiotics pollution. The results support the use of lysosomal membrane destabilization together with lysosomal enlargement as a prognostic biomarker for toxicant-induced fish liver dysfunction in biomonitoring programs (Broeg et al., 1999; Köhler et al., 2001, 2002). Methods used in the determination of lysosomal integrity in fish hepatocytes have been well established (Köhler 1991, 1992; Lowe et al., 1992), but no information is available on the reversibility of lysosomal changes upon withdrawal of contaminants. If the induction is transient and reversible, this cytological damage is short term, and induction of the cytological changes will signify recent exposure of the fish to pollutants. If the change is irreversible, permanent damage is implicated. Such information is essential for interpreting field data and evaluating the robustness of these cytological markers for pollution monitoring and risk assessment.

Both lysosomal stability and lipopigment content in fish liver have shown promise and may be useful early warning signals to indicate exposure to and effects of persistent environmental pollutants, providing that the effects of seasonal variations (temperature, salinity, and food availability) and reproductive status (sex and maturity) have been studied and standardized for the test fish species. Such information is urgently needed for evaluating the applicability of the two cytological biomarkers in pollution monitoring. The fact that major attention in biomarker production and application has focused on the liver underscores the importance of this target organ.

Need for Future Research

More data on responses of fish liver to hepatotoxicants are needed. We have a number of reports describing toxic responses in fish liver, but usually only specific aspects are addressed, and rarely do we have a database comparable to reference hepatotoxicants in mammals, where information regarding the mechanisms, pathology, and toxicology is available. With fish, we usually have only bits, but no complete picture. For rodent liver, we are aware of pathological liver changes typical for, for example, oxidative stressors, and we can even cluster hepatotoxins on the basis of gene expression profiles, but for fish we have no such systematic understanding. To broaden our database on this point requires generating a database on the mechanisms and pathological and toxicological effects of chemicals that act specifically on the liver (not just chemicals that may target another organ and have some side effects on the liver as well), which is essential for developing our still very descriptive approach to fish liver toxicology into one of sound and integrative science.

In addition to broadening our knowledge and understanding on toxic response patterns, both molecular and histological ones, we have to know much more regarding the basic functional properties of fish liver; for example, although the various sinusoidal and biliary transporters in rodent liver are well characterized, we are just beginning these studies in fish. We have some evidence for an MDR1-like transporter, and evidence is emerging for MRP-like transporters, but all this is still very fragmentary information. As long as we lack sufficient understanding of the basic functional traits of fish liver, we will not be able to understand and classify toxic effects.

We need to know much more regarding the relationships between the various liver cell types. Much liver toxicity in mammals is not triggered from hepatocyte responses but through signaling between the various liver cell types. Here, our knowledge on fish liver is almost nonexistent, despite the importance of this aspect for liver toxicology. Currently, liver histopathology is used mainly as a descriptive tool to assess the health status of fish exposed to toxicants. To more completely understand toxic responses in the liver and to further apply this knowledge, we need to integrate molecular, biochemical, physiological, and morphological findings from the cellular through organ level.

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8

The Osmoregulatory System

Sjoerd E. Wendelaar Bonga and Robert A.C. Lock

CONTENTS

Introduction

The gills and the gut are the main entry routes for toxic agents in teleost fish, and this makes these organs important targets for such chemicals. Gills and gut are also important organs for the regulation of water and ions in fish, and this is the main reason why many toxic agents, either waterborne or present in the food, are well known for disturbing hydromineral regulation in fish. This is readily reflected in changed osmolarity and the concentration of the main ions (in particular Na^+, Cl^-, Ca^{2+}, and Mg^{2+}) in the blood plasma. Effective hydromineral control is of utmost importance in fish, as most of these animals are living either in freshwater, with an osmolarity in general less than 5 mOsmol L^{-1} and thus far below that of the blood plasma (300 to 325 mOsmol L^{-1}), or in seawater, with an osmolarity about three times higher than in the blood of seawater-dwelling fish (950 to 1050 mOsmol L^{-1}). The resulting osmotic and ionic gradients across the branchial epithelia form the driving force for passive water and ion movements between the fish and the water. These movements—the outflow of ions and osmotic uptake of water in freshwater fish and the inflow of ions and osmotic water loss in seawater fish—are counteracted by an effective control of permeability to water and ions of the integumental epithelia, particularly in freshwater conditions (Eddy, 1981; Evans, 1987). Nevertheless, compensation of the resulting water and ion flows requires a constant action of the principal water and ion regulatory organs, the gills, skin, and kidneys, in both freshwater and seawater. The functions of the three organs differ substantially between freshwater and seawater fish, and this is one of the reasons why the impact of many toxic agents is dependent on water salinity. In freshwater, the outflow of ions over the gills is offset by active ion-exchange processes in the gills and intestine, whereas the osmotic inflow of water is compensated by the excretion of large volumes of urine with a low osmolarity. In a marine environment, the osmotic loss of water is compensated by drinking seawater and the subsequent secretion of monovalent ions via the gills and monovalent and divalent ions via the kidneys. The latter processes also redress the passive inflow of ions in seawater fish.

The exchange and secretion of ions are essential not only for compensating the passive ion flows between fish and the ambient water and the excretion of superfluous ions extracted from food, but also for the elimination of metabolically produced ions, such as H^+, NH_4^+, and HCO_3^-. The gills have a particularly important role in this respect, as they function as organs for the exchange of ions and respiratory gases as well as for acid–base regulation (Evans, 1987; McDonald et al., 1991). Thus, when considering the actions of toxic agents on hydromineral regulation in fish, the effects of these substances on gills, gut, and kidneys have to be taken into account.

In this chapter, we review the most important aspects of the interactions of toxic agents with these osmoregulatory systems on the basis of a selection of the available literature and with an emphasis on those aspects that are typical for fish as aquatic organisms. We discuss the effects of direct interaction of toxic agents with the osmoregulatory epithelia, as well as the indirect effects resulting from the actions of toxic chemicals as stressors and as disruptors of endocrine cells. Like all external factors with the capacity to disturb internal equilibria of animals or with an impact experienced as threatening, toxic agents can evoke a stress response in animals. In fish, much more than in the terrestrial vertebrates, stressors affect the hydromineral balance of the fish. This is an aspect of fish that has long been ignored or underestimated in studies on toxic mechanisms and toxic effects in these animals (Wendelaar Bonga, 1997).

The Gills

We consider here the direct and indirect effects of toxic agents on the hydromineral and acid–base balance that can be attributed to damage of the branchial structure and alterations in branchial function. Gills consist of two sets of four branches, with numerous rows of filaments; each filament has two rows of lamellae, which represent the respiratory surface. The afferent arteries to the gills open into blood sinuses in the lamellae—flat spaces lined by cytoplasmic extensions of the pillar cells. The spaces are drained by efferent arterioles, which anastomose with a central venous sinus in the filament, drained by venolymphatic vessels (Figure 8.1). Whereas the lamellae are covered by a uniform one- to two-layer flat epithelium of respiratory cells, the filament epithelium is multilayered and consists mainly of filament cells, with an apical layer of flattened pavement cells with mucus cells and chloride cells. The chloride cells are the main location of ion-dependent ATPase activity as well as ion channels and other mechanisms involved in transcellular and paracellular ion transport. There are notable indications that H^+–ATPase activity is also present in respiratory cells and/or pavement cells (see reviews by Evans, 1987; Flik et

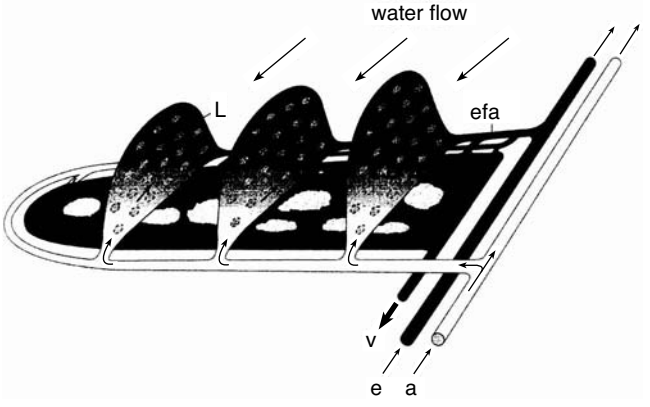

FIGURE 8.1 Simplified diagram of a branchial filament with lamellae (L; their numbers vary from 30 to several hundred per filament, depending on species). The blood enters the filament via a branch of the afferent gill arch artery (a) and enters the vascular spaces of the lamellae. These are drained by efferent lamellar arteries, which join the efferent filament artery (efa), which opens into the efferent arch artery (e). Part of the blood enters the central venous sinus of the filament, which is drained by a venolymphatic vessel (v).

al., 1996; McDonald et al., 1991). The large respiratory surface of the lamellae as well as the extensive epithelium lining the filaments represent an important area of contact between the animals and the ambient water which facilitates an efficient exchange of respiratory gases and ions but at the same time forms an expanded and delicate target area for toxicants.

Numerous reports have been published on the effects of toxic agents on gill structure and function, mainly in freshwater fish, although interest in seawater fish is growing. Mallatt (1985) and Evans (1987) summarized the main literature on gill structure available in the mid-1980s, and they emphasized the diversity of toxic actions as well as similarities in the types of lesions induced by a large number of toxicants. Common structural alterations include necrosis of pavement cells and chloride cells; rupture of the epithelium; hypertrophy and hyperplasia of chloride cells, filament cells, and respiratory cells which may lead to lamellar fusion; epithelial swelling by widening of intercellular spaces; penetration of leucocytes from the blood into these intercellular spaces; and, in the lamellae, epithelial lifting, the separation of the respiratory epithelium from the underlying tissue. Such alterations have been confirmed for many toxicants (Grinwis, 1998; Nowak, 1992). More recently, several studies have shown that cell death by toxic agents is due not only to necrosis (accidental cell death) but also apoptosis (physiologically controlled cell death) (Wendelaar Bonga, 1997; Bury et al., 1998). In this chapter, we mainly concentrate on the literature after 1987 and focus on the underlying action mechanisms and consequences for the hydromineral balance. The effects of toxicants on the gills are twofold: (1) interaction (mainly inhibition) with the ion-transporting mechanisms of the gills, which are mainly concentrated in the chloride cells, and (2) increased permeability of the branchial epithelia to water and ions. Although the gills are mainly affected by waterborne chemicals acting from outside, bloodborne chemicals that have entered the circulation via the gut have also been reported to disturb branchial structure and function (Handy, 1992; Pratap and Wendelaar Bonga, 1993).

Disturbance of Active Ion Transport Processes

The chloride cells are the most extensively studied cell types of the gills because of their key function in ion transport activity. They are characterized by an extensive tubular system continuous with the basolateral membrane and containing membrane-bound, ion-translocating enzymes such as Na^+/K^+- and Ca^{2+}-ATPase, as well as different types of exchangers and postulated ion channels in the apical and basolateral membranes (Figure 8.2). Exposure to toxicants, particularly heavy metals, leads to a rapid inhibition of these cells. In general, at high concentrations, this is structurally reflected by focal damage or by necrosis and apoptosis of these cells. In response to cell death, differentiation of new cells is commonly observed. The acceleration of cell death and cell replacement may continue for months, although its rate in general slows down after the first week, possibly as a result of the expression in the newly formed cells of proteins such as metallothioneins (Roesijadi, 1996), which may protect the cells against the toxic action of the metals.

Chloride cells can be affected by waterborne as well as dietary metals, as has been shown for cadmium in Mozambique tilapia (Pratap and Wendelaar Bonga, 1993) or copper in trout (Berntssen et al., 1999). The toxic mechanisms involved have been elucidated for only a few metals. Copper, which is known to affect plasma Na^+ ions and Na^+ fluxes rather specifically (Laurén and McDonald, 1987; Li et al., 1998; Matsuo et al., 2004), has been shown to inactivate Na^+/K^+-ATPase activity (the driving force for Na^+, K^+, and NH_4^+ transport and indirectly for other ions such as H^+, Ca^{2+}, and, in seawater, Cl^-) *in vitro* in the nanomolar range (Li et al., 1996). The result is a net loss of sodium and other ions.

The differences among species with regard to sensitivity to copper (e.g., yellow perch is rather tolerant and rainbow trout is sensitive) have been attributed to the rate of sodium loss upon copper exposure and not to the total amount of copper bound to the gills, which are the sites of highest copper accumulation in fish. Copper accumulation was found to be 9 times higher in perch than in trout (Taylor et al., 2003). At copper concentrations causing 50% mortality (96-hr LC_{50}), cadmium is able to inhibit *in vitro* Ca^{2+}-ATPase activity, the driving force for branchial and intestinal Ca^{2+} uptake, in the nanomolar range (Schoenmakers et al., 1992). This seems to be the key toxic mechanism for this metal. Reductions in plasma Ca^{2+} concentrations after exposure to cadmium have been reported for several fish species. Conversely, an increase in waterborne Ca^{2+}, but also increased dietary calcium intake, can reduce the

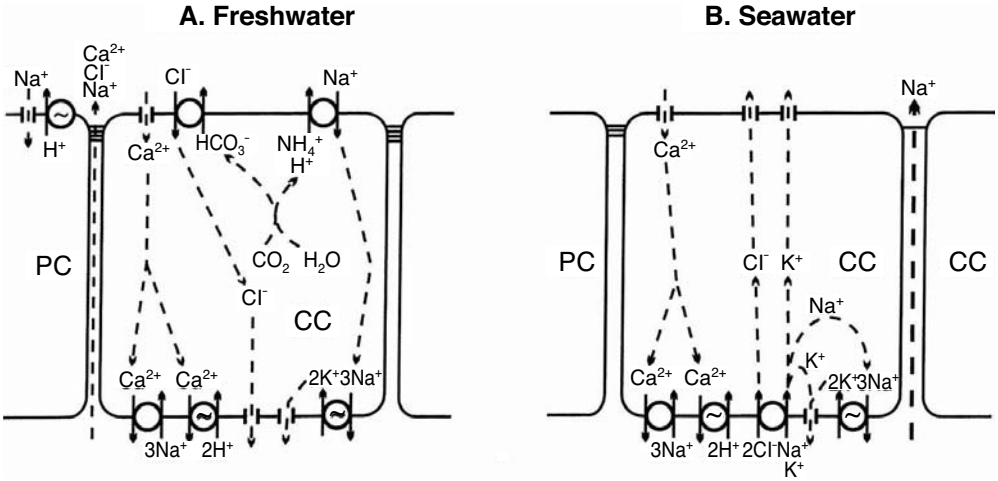

FIGURE 8.2 Diagrammatic representation of chloride cell function in freshwater fish (A) and seawater fish (B). Active transport (~) and cotransport are indicated by solid lines and diffusion by dashed lines. Tight intercellular functions are indicated as multi-stranded lines; leaky junctions as single-stranded lines. This leaky junction (in seawater) permits Na efflux driven by serosa-positive transepithelial potential. PC, pavement cell; CC, chloride cell. (Adapted from Marshall, W.S., in *Fish Physiology*, Vol. XIV, Wood, C. M. and Shuttleworth, T. J., Eds., Academic Press, San Diego, CA, 1995, 1–23; Flik, G. et al., *Physiol. Zool.*, 69, 403–417, 1996.)

toxicity of both waterborne and dietary cadmium by reducing the uptake through gills and gut (Baldisserotto et al., 2005). The uptake of inorganic mercury can be partially inhibited by the sodium-channel blocker phenamel, but not by calcium-channel blockers, indicating the possibility of competition by mercury for Na^+ uptake (Klinck et al., 2005).

For several other metal ions, the toxic mechanism has not been defined clearly. The gills are the tissue with the highest metal accumulation after exposure to waterborne lead. Inhibitory effects of lead on branchial Na^+/K^+-ATPase activity and Na, Cl, and Ca^{2+} influx have been reported for rainbow trout, as well as reduced plasma Na^+, Ca^{2+}, and Cl^- levels. The acute toxicity for lead is probably connected with ion regulatory disruption rather than, for example, respiratory failure or acid–base disturbance, but the key toxic mechanism is still unclear (Rogers et al., 2003). Waterborne nickel has no clear effect on Na^+, Cl^-, or Ca^{2+} fluxes and, in contrast to most other metals, is most likely a respiratory rather than an ion regulatory toxicant (Pane et al., 2003). At high concentrations, the plasma levels of many other ions are affected, and this can be ascribed to the destruction of the chloride cells. At sublethal exposure levels, this reduction is transient, probably as a result of replacement by more resistant newly differentiated chloride cells. Currently, no specific effects of organic toxicants on the ion-transporting mechanisms of chloride cells have been determined. Most likely, the malfunctioning of chloride cells by organic toxicants is the result of nonspecific damage to the gill epithelium or to damage of the regulatory mechanisms of these epithelia.

When examining the effects of toxicants *in vivo*, two processes interfere with each other: the toxic actions of the chemicals themselves and the compensatory responses of the fish. This interference complicates the analysis of the effects of toxic substances. Successful compensation may obscure the negative effects of toxicants and lead to an underestimation of their impact on living organisms, as compensatory processes require energy. The allocation of energy toward compensatory processes and cell-cycle acceleration limits the potential growth and reproduction of organisms and promotes aging. Compensatory processes also impair the analysis of action mechanisms, which may explain many discrepancies reported in the literature; for example, reports on the effects of metals on Na^+/K^+-ATPase activity or total number of chloride cells show many inconsistencies. Cadmium exposure induces an increased turnover of chloride cells. Depending on the balance between cell death and cell replacement, which in turn is dependent on cadmium concentration, the presence of other stressful factors, and the exposure time, the numerical density of these cells may decrease, remain constant, or increase (Wendelaar

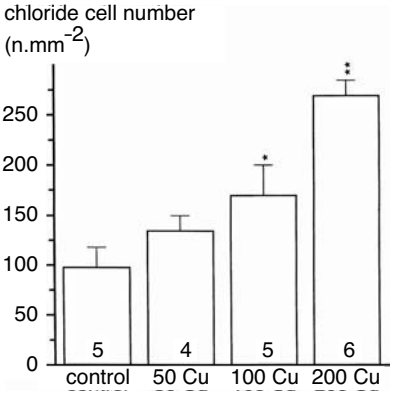

FIGURE 8.3 Chloride cell numbers in the gill opercular membrane (reflecting total gill epithelial surface area) of the Mozambique tilapia (*Oreochromis mossambicus*) exposed for 6 days to 0 (control), 50, 100, or 200 µg Cu/L. Means ± SD are indicated; numbers of fish per group are shown in the bars; $*p < 0.01$; $**p < 0.001$. (From Pelgrom, S. M. G. J. et al., *Aquat. Toxicol*., 32, 303–320, 1995. With permission.)

Bonga and Lock, 1992). Discrepancies among various studies with regard to the effects of toxicants on the Na^+/K^+-ATPase activity of gills may have a similar causes. Inhibition of Na^+/K^+-ATPase activity by, for example, copper may be masked by overcompensation by the fish through active stimulation of the formation of new chloride cells (Figure 8.3).

Disturbance of Permeability to Water and Ions

As mentioned above, maintenance of the membrane permeability to water and ions is the basis for controlling the hydromineral balance in fish. Any factor interfering with branchial permeability poses a serious threat to fish, because, at least in freshwater fish, this leads to reductions in plasma ion levels and, subsequently, ion losses from the intracellular compartments. Several types of toxic agents have direct effects on branchial permeability. Copper, for example, has been reported to increase the effluxes of Na^+, Cl^-, and K^+ (Laurén and McDonald, 1985; Reid and McDonald, 1988); silver increases Na^+ efflux (Bury, 2005); zinc and lanthanum stimulate Na^+ and Cl^- effluxes (Spry and Wood, 1985); cadmium promotes Ca^{2+} efflux (Verbost et al., 1987); and lead promotes diffusional water fluxes (Sola et al., 1994). The transcellular permeability of an intact epithelium is mainly determined by the characteristics of its membrane phospholipids and the amount of calcium bound to the negative groups of the membranes, whereas the permeability of the paracellular routes is dependent on the structure of the tight junction interconnecting the apical areas of the upper cell layers. Loss of calcium from the membranes and tight junctions of fish gills (e.g., as a result of replacement by heavy metals) has been considered an important cause of increased branchial permeability (McDonald et al., 1991; McWilliams, 1982). The protective effect of high water calcium levels against heavy metal toxicity is in agreement with this hypothesis (Pratap et al., 1989), although water Ca^{2+} may also reduce metal uptake by competing with metals for calcium channels (Comhaire et al., 1998). Extensive epithelial lesions may also affect epithelial permeability to water and ions; this could be an additional mechanism for heavy metals and perhaps the main mechanism for the (relatively few) organic pollutants with direct actions on water and ion regulation, such as perfluorooctane sulfonic acid (PFOS). This chemical is a wetting and foaming agent that reduced serum Cl^-, Na^+, and Ca^{2+} levels in carp, probably through disruption of membrane structure in the gills (Hoff et al., 2005). Branchial permeability to water and ions can also be increased indirectly, via the induction of a stress response.

A rapid rise in the blood levels of adrenaline and noradrenaline is typical of a stress response, which is evoked by such factors as toxic chemicals that threaten or disturb the physiological homeostasis of animals. High adrenaline levels increase the blood pressure in the gills, as a result of increased cardiac output and arterial vasoconstriction. Lamellar perfusion is increased by adrenaline, via opening of the

afferent lamellar vessels and closing of the arteriovenous anastomoses (Figure 8.1). The increased blood flow and the recruitment of peripheral branchial lamellae that are barely perfused at rest increase not only the effective respiratory surface but also the available surface area for the diffusion of water, ions, and even large organic molecules (Mazeaud and Mazeaud, 1981; Randall and Perry, 1992). As a result, all factors stimulating this adrenaline release also promote the passive loss of ions in freshwater fish and the passive inflow of ions in seawater fish across the gills. In freshwater fish, diffusive losses of Na^+ and Cl^- can increase up to 40-fold during stress, resulting in a dramatic loss of total body Na^+ and Cl^- and further promoting the osmotic flow of water across the gills (see reviews by Mazeaud et al., 1977; McDonald and Milligan, 1997).

Factors affecting the release of adrenaline vary from forced increased locomotor activity or a reduction in water oxygen concentration to all kinds of social, physical, or chemical stressors. All toxic chemicals increase branchial permeability to water and ions as soon as their impact is sufficient to evoke a stress response, and severe stress can lead to edematous swelling of the lamellar and filamental epithelia and even to the phenomenon of epithelial lifting (detachment of the lamellar epithelium from the underlying tissues). As mentioned earlier, such phenomena have frequently been described in fish exposed to inorganic or organic pollutants (Mallatt, 1985; Wendelaar Bonga, 1997). More direct evidence that these chemicals can produce a stress response are the many reports on the elevation of plasma cortisol levels following exposure of fish to these substances (Hontela, 1997; Pelgrom et al., 1995; Webb and Wood, 1998). Recently, Chowdhury et al. (2004) reported increased levels of plasma cortisol, glucose, lactate, and total ammonia, all classical stress parameters, in rainbow trout exposed to waterborne cadmium. The observed rise in aspartate aminotransferase and alanine aminotransferase in gills, kidneys, and liver of common carp during cadmium exposure has also been ascribed to the stress response evoked by this metal. Both enzymes are known to be involved in protein catabolism for energy production in stressed animals (De Smet and Blust, 2001).

The recovery phase of the stress response is characterized by a compensatory increase of chloride cell density in the gills. This explains why not only toxic metals that directly affect these cells but also organic chemicals without specific effects on the gills increase chloride cell density (Mallatt, 1985; Wendelaar Bonga, 1997).

The Intestine

The intestine is one of the main targets of dietary and waterborne toxic agents, and, given the important osmoregulatory function of the intestine, it directly affects the regulation of water and ion balance. It is therefore surprising that the intestine, as an organ of hydromineral regulation, has received little attention from fish toxicologists, particularly when compared to the hundreds of papers on the gills. The main reason may be that most toxicological studies are dealing with the effect of waterborne pollutants on freshwater fish, which have low drinking rates. It should be emphasized here that, under field conditions in neutral water, toxic metals are almost exclusively complexed or bound to particles and enter the fish mainly as food contaminants.; thus, for reasons of ecological relevance, more attention should be paid to the intestinal route as far as the toxicology of metals is concerned.

In freshwater fish, the role of the intestine in the control of hydromineral balance is comparable to that of the terrestrial vertebrates, with reabsorption of calcium and magnesium being the most important activity, complementary to the mineral-accumulating function of the gills (Flik et al., 1996). The situation in seawater is essentially different. The high drinking rate of seawater fish, followed by intensive reabsorption of water to offset the osmotic water loss, is an important strain on hydromineral control as the water reabsorption is dependent on Na^+ and Cl^- uptake. The role of the intestine in hydromineral regulation is reflected by the structure of the epithelial cells. The dominating cell type, the enterocyte, exhibits apically the appearance of cells involved in nutrient reabsorption, such as a well-developed brush border and a well-developed lysosomal system, in addition to smooth and granular endoplasmic reticulum and an occasional peroxisome. Along the entire length of the intestinal tract, however, particularly in its more rectal parts, the basal parts of these cells show the typical characteristics of cells involved in the transepithelial transport of water and ions: a basal labyrinth of folded

membranes forming slit-like lumina communicating by pores with the extracellular space at the serosal side of the epithelium, with many elongated mitochondria between these folds. The membranes are the probable location of ion-translocating mechanisms that have been demonstrated in the intestine: Na^+/K^+-ATPase, Ca^{2+}-ATPase, and a Na^+/Ca^{2+} exchanger (Schoenmakers et al., 1993). In the brush border of the enterocytes of Mozambique tilapia, a low-affinity Ca^{2+} transporting carrier has been demonstrated. Ample evidence for transcellular and paracellular ion transport has been presented (see review by Flik et al., 1996).

The structure of the enterocytes is essentially similar in freshwater and seawater fish, although the basal labyrinth is more extensive in the latter. This reflects the high intensity of the intestinal reabsorption of water, Na^+, and Cl^- in marine fish that is required to offset the osmotic loss of water across the integument in seawater; however, an important qualitative difference is the presence of the $Na^+/K^+/2Cl^-$ cotransporter in seawater fish.

The more than tenfold difference in drinking rate between freshwater and seawater fish has important consequences for the exposure of the intestine to toxic agents. Whereas in freshwater it is mainly the pharyngeal area that is in contact with the ambient water as a result of the respiratory water movements, in seawater the entire alimentary tract is exposed, particularly the rectal part, where most of the water and ions are taken up. Thus, in seawater, waterborne toxicants are potentially more threatening to fish than in freshwater; however, a direct comparison is complicated by the differences in bioavailability of toxic agents that may occur as a result of salinity effects on speciation of the toxicants. This holds in particular for toxic metals.

Heavy Metals

From the point of view of hydromineral control, the actions of heavy metals on the intestine are comparable to those on the gills, as they affect inhibition of ion-transporting mechanisms and increase the permeability of the intestinal epithelium. Reduction of intestinal Na^+/K^+-ATPase activity has been reported for several metals, including mercury (Lakshmi et al., 1991), chromium (Boge et al., 1988; Thaker et al., 1996), and cadmium (Schoenmakers et al., 1992). Mozambique tilapia has been shown to become hypocalcemic when fed cadmium-containing food (Pratap et al., 1989). Apparently, these fish could not compensate the decrease of intestinal calcium uptake by stimulation of branchial uptake from the water. This may be the result of dietary cadmium entering the blood and inhibiting branchial calcium uptake, as was shown by Baldisserotto et al. (2005) for rainbow trout. Conversely, these authors also showed that an increase of dietary calcium reduced both intestinal and branchial cadmium uptake. In tilapia, increased apoptosis and necrosis of chloride cells has been reported for Mozambique tilapia exposed to dietary cadmium, similar to what occurs after exposure to waterborne cadmium (Pratap et al., 1989). The hypocalcemia induced by cadmium already at low concentrations may be partially caused by inhibition of intestinal calcium uptake. Schoenmakers et al. (1992), studying intestinal basolateral membrane preparations of Mozambique tilapia, showed that Cd^{2+} inhibits the active transport of Ca^{2+} across these membranes, probably by inhibiting the Ca^{2+}-ATPase activity in these membranes. The same authors, studying the effects of Cd^{2+} on stripped intestinal epithelium of the same species in an Ussing chamber, further demonstrated that the addition of Cd^{2+} at the mucosal, but not serosal, side decreased the active transcellular Ca^{2+} transport across this epithelium (Schoenmakers et al., 1992). The ATP-driven calcium translocation across these membranes was extremely sensitive to cadmium, indicating inhibition via the calcium site of the enzyme (Figure 8.4). Intestinal Na^+/K^+-ATPase activity was half maximally inhibited at 2.6 μM Cd^{2+}, with cadmium probably competing for the Mg^{2+} site of the enzyme. Thus, the sensitivity for Cd^{2+} of both enzymes differs by five orders of magnitude. The Na^+/Ca^{2+} exchanger was only partially blocked by cadmium binding. Finally, the mucosal and serosal addition of Cd^{2+} resulted in increased permeability to Ca^{2+} of the paracellular route in this epithelium, and this may result in passive losses of Ca^{2+} in freshwater or the inflow of Ca^{2+} in seawater.

In an electrophysiological study on seawater eels, evidence was obtained that Cd^{2+} affects intestinal Na^+ and Cl^- reabsorption via both cellular and paracellular mechanisms. In marine fish, the intestinal Na^+ and Cl^- reabsorption is affected by the luminal $Na^+/K^+/2Cl^-$ cotransporter in conjunction with serosal Na^+/K^+-ATPase activity and Cl^- conductance (Trischitta et al., 1992). Cd^{2+} blocked Na^+/K^+-ATPase

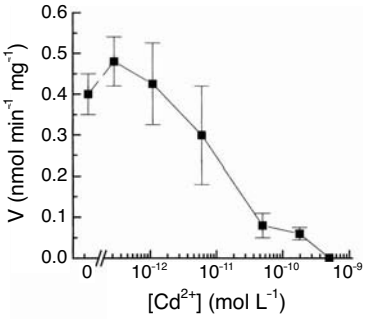

FIGURE 8.4 Cd^{2+} inhibition of the ATP-dependent calcium pump in basolateral plasma membrane vesicles from the intestinal epithelium of Mozambique tilapia *(Oreochromis mossambicus)*. Means ± SEM of nine experiments are given. The Ca^{2+} concentration was kept constant at 200 n*M*. Initial rates of ATP-dependent Ca^{2+} uptake were corrected for ATP-independent uptake. The calculated IC_{50} is 8.2 ± 3.0 p*M* Cd^{2+}. (From Schoenmakers, T. J. M. et al., *J. Membr. Biol*., 127, 161–172, 1992. With permission.)

activity and possibly also the cotransporter and affected the cation selectivity of the tight junctions. Because Na^+ and Cl^- absorption is the main driving force for intestinal water uptake, these effects of Cd^{2+} seriously threaten the water balance of marine fish (Lionetto et al., 1998).

When considering the toxicity of metal ions in a marine environment, it should be kept in mind that speciation effects, such as the formation of metal chlorides, drastically reduce metal toxicity. For cadmium, only a very small fraction is present as Cd^{2+} in seawater. In the intestine, cadmium is readily bound to the organic material present in this organ. Marked intestinal accumulation of waterborne cadmium and copper has been reported for freshwater fish (Handy, 1992). Because the low drinking rate in these animals could not account for this accumulation, the authors suggested a special role for the intestine as a store and excretion route for these metals, as suggested before for rats (Stonard and Webb, 1976). At high concentrations, cadmium may disrupt the mucosal folds in the intestine, as reported by Ghosh and Chakrabarti (1992) for *Notopterus notopterus*. These authors reported that the enterocytes were losing their regular arrangement and became necrotic, whereas the mucus secretion was stimulated. Such histopathological changes in fish intestine have also been reported for copper (Bakshi, 1991). In a study on Atlantic salmon, Lundebye et al. (1998) have shown that dietary copper and cadmium at high concentrations (up to 700 and 5 mg kg^{-1} diet, respectively) induced necrosis as well as apoptosis of enterocytes. Tissue necrosis may be accompanied by enhanced permeability of the epithelium to water and ions.

Although a direct comparison of exposure to waterborne and dietary heavy metals is complicated, dietary exposure to copper or cadmium is generally considered less toxic than exposure via the water (Lanno et al., 1985; Miller et al., 1993). This has been ascribed to the characteristics of the intestinal epithelium, which apparently represents a more effective and less sensitive barrier to toxic metals than the branchial epithelium (Handy, 1993). The complexation of metals with the organic contents of the gut, as mentioned above, may be another mitigating factor.

Organic Pollutants

No studies focusing on the effects of organic pollutants on the hydromineral functions of the fish intestine are known to the authors. Interference with these functions can only be deduced from the disruptive effects reported on the structure of the intestinal tracts and from a few studies on intestinal ion-dependent ATPase activity. Spitsbergen et al. (1988), for example, reported submucosal gastric edema and epithelial necrosis in perch and rainbow trout treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Such effects, reported for many organic pollutants, would affect the water and ion balance as a result of nonspecific damage inflicted to the intestinal epithelium and, more indirectly, via the induction of a stress response. Chronic stress in fish, as in tetrapods, is known to be accompanied by gastric and intestinal lesions (Peters, 1982; Szakolczai, 1997).

The Kidneys

The multifunctional role of the fish kidney, which combines the functions of lungs, kidneys, and intestines of terrestrial vertebrates, is reflected by its complicated structure involving many different cell types and an intricate vascular system. From the point of view of hydromineral regulation, the main function of the kidneys in freshwater fish is the excretion of large volumes of osmotically accumulated water and the elimination of surplus ions extracted from the food, mainly K^+, sulfate, and phosphate. To limit the renal losses of valuable ions via the urine, intensive reabsorption of Na^+, Cl^-, Mg^{2+}, and Ca^{2+} takes place by means of transporting enzymes and exchangers in the epithelial cells lining the nephronic tubules, collective tubules, and bladder. In seawater fish, the excretion of divalent ions taken up by drinking is a major function of the kidneys. Water excretion has to be reduced as much as possible in a strongly hyperosmotic environment, and this is effected mainly by reduction of the glomerular filtration rate (Beyenbach, 1995). The basal parts of all the cells in the renal tubular epithelia as well as the lining of the bladder have essentially the same structure: a basal labyrinth consisting of membranes and mitochondria, with the same structure as the basal areas of the intestinal cells. In freshwater fish, the extent of the membranes and density of the mitochondria increase with the distance of the cells from the glomeruli, with maximal development of the basal labyrinth occurring at the end of the collecting tubules and in the bladder. In seawater fish, the basal labyrinth is less well developed and a gradient is usually not noticeable (Wendelaar Bonga, 1973).

Toxicological studies have revealed extensive histopathological alterations in the kidney as a result of exposure of fish to heavy metals and organic chemicals. In general, the structural damage to the kidneys seems to show little toxicant specificity. The proximal tubules appear to be the first affected, but damage progresses to the posterior segments and glomeruli, following exposure of the fish for a longer period or to a higher concentration of the toxicant. Histopathological effects vary from slight disruption of the brushborders of the proximal tubular cells, increased numbers of phagosomes and lysosomes, and structurally abnormal mitochondria to rupture of cellular membranes, swelling and vacuolization, increased cell death by necrosis and apoptosis, and large lesions in the tubular epithelia (Gill et al., 1989; Oulmi and Braunbeck, 1996; Sovenyi and Szakolczay, 1993). Cadmium is well known for its preference for renal tissue as a site of accumulation; the highest concentrations of this metal, after water as well as dietary exposure, are usually found in the kidneys (Bentley, 1991; McCoy et al., 1995; Thomann et al., 1997). As a consequence, cadmium is able to produce severe histopathological alterations (Gill et al., 1989; Ooi and Law, 1989; Oronsaye, 1989; Sovenyi and Szakolczay, 1993). Although copper has no specific affinity for the kidneys, it can also produce significant structural damage to both glomeruli and renal tubules (Bakshi, 1991). Among the many organic chemicals producing similar effects are paraquat (Molck and Fries, 1997), 4-chloroaniline (Oulmi and Braunbeck, 1996), TCDD (Henry et al., 1997), cyclosporin (Terrero and Coombs, 1989), and atrazine (Fischer-Scherl et al., 1991).

Given the severity of the histopathological changes it is clear that renal functions, including hydromineral regulation of the kidneys, will be affected with a severity depending on the concentration and length of exposure. Among the relatively few studies dealing with renal functions are reports on the inhibitory effects of silver on K^+-dependent *p*-nitrophenol phosphatase (Bury, 2005) and of cadmium, chromium (Venugopal and Reddy, 1993), copper, and mercury (Lan et al., 1993) on renal tubular Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Na^+/Ca^+ exchange activity. The mechanisms of action may be similar as described for the gills and intestine: specific interactions with the ion-transporting molecules at lower concentrations and lethal cell damage at higher concentrations via induction of cellular necrosis or apoptosis. The latter, less specific way of reduction of renal ion transport activity may be typical of most organic pollutants. The reduction of Na^+/K^+-ATPase activity induced by paraquat has been related to the disruption of this chemical on mitochondrial electron chain transfer, which results in reduction of metabolic functions (Molck and Fries, 1997). The nephrotoxicity of cyclosporin is based primarily on its action as a calmodulin inhibitor, and its effects on renal hydromineral regulation have been ascribed to interference with calmodulin-dependent ion transport mechanisms (Terrero and Coombs, 1989).

An interesting finding on copper was the decreased renal Na^+ efflux of freshwater rainbow trout following exposure to waterborne copper. Given the strong inhibitory action of copper on Na^+/K^+-ATPase

activity (Li et al., 1996), the opposite effect was expected, as reduction of Na^+ efflux points to increased tubular reabsorption of Na^+, for which Na^+/K^+-ATPase is a driving force. This phenomenon was explained as a physiological response of the kidneys to compensate the impairment of branchial Na^+ uptake (Grosell et al., 1998). This indicates that the renal Na-/K^+-ATPase activity provides better protection against copper than the branchial activity, and this is in agreement with the low renal accumulation and excretion rates for copper. Few studies are available on the physiological aspects of renal hydromineral function such as urine flow and renal ion excretion rates. Collection of such data requires cannulation techniques that are complicated and, because they are extremely stressful to the fish, often lead to results that are biased by the experimental procedures; thus, a proper evaluation of the effects of toxic chemicals on the kidneys is even more complicated than on gills and intestine.

Toxic Agents and Endocrine Control of Osmoregulation

Good evidence from fishes suggests that toxic agents can affect neuroendocrine cells and in this way interfere with neuroendocrine control processes, including osmoregulation (Hontela et al., 1993). The cell types that have received most attention in this respect are the prolactin cells and adrenocorticotropic hormone (ACTH) cells in the pituitary gland and the interrenal cells producing cortisol. Prolactin is an important hormone for the control of permeability to water and ions in the gills, intestine, and renal tubules. Activation of prolactin cells has been frequently observed in fish exposed to toxic agents and has been generally interpreted as a compensatory response to the disturbance in the permeability characteristics of these epithelia by toxicants (see review by Wendelaar Bonga and Pang, 1989). ACTH is an important secretagog of cortisol (Balm and Pottinger, 1999). The latter hormone is not only one of the primary stress hormones in fish (see under Gills) but also an important stimulator of Na^+/K^+-ATPase activity in gills, intestine, and kidneys. The multiple functions of this hormone clearly demonstrate the intimate relationship between the stress response and osmoregulation in fish. These functions of cortisol explain the stimulation of cortisol release that has been frequently reported for fish exposed to toxic agents (see reviews by Hontela, 1997; Wendelaar Bonga, 1997).

Norris et al. (1997) reported that the hypothalamo–pituitary–interrenal (HPI) axis of feral brown trout is activated in fish from sites contaminated with cadmium and zinc. They reported a higher number of immunoreactive neurons in the hypothalamus than in fish from control sites and further observed hypertrophy and hyperplasia in the interrenal cells of the fish from contaminated water; however, the relationship between some toxic agents and cortisol secretion may be more complicated and includes interference with the secretion of cortisol and ACTH. The interrenal tissue of yellow perch from a site contaminated by a mixture of heavy metals and organic contaminants secreted significantly less cortisol in response to a standardized pulse of ACTH than the interrenal tissue of fish from an unpolluted reference site (Brodeur et al., 1998).

Field studies on the effects of a mixture of contaminants (including heavy metals and PCBs) showed endocrine dysfunction in fish from natural water contaminated with a mixture of pollutants when compared with fish from an unpolluted reference site (Hontela et al., 1992, 1995). Feeding low levels of PCB 169 to rainbow trout stimulated and higher levels impaired the production of cortisol, cortisone, and other metabolites by interrenal cells as determined *in vitro* (Freeman et al., 1984). Short-term exposure (5 days) of Mozambique tilapia to PCB 126 did not influence branchial, renal, or intestinal Na^+/K^+-ATPase activity, nor did it evoke a stress response, as was concluded from the absence of a rise in plasma cortisol or glucose levels; however, when the fish were exposed to acute sampling stress, plasma cortisol levels were lower in PCB-fed fish than in controls (Figure 8.5). The interrenal cells of PCB fed fish proved to be less responsive to ACTH or cAMP stimulation *in vitro*, indicating direct toxic effects on the interrenal cells, rather than secondary effects via disturbance of hydromineral imbalance or stimulation of mixed-function oxygenase systems involved in steroid catabolism. (Quabius et al., 1997). Vijayan et al. (1997) observed an impaired ability to elicit a cortisol response in stressed trout exposed to PCB 77 and suggested that this might be caused by the enhanced hepatic cortisol clearance in these fish. Given the importance of cortisol for osmoregulation in fish, any interference with the secretion of cortisol and its secretagogs such as ACTH may be expected to have an effect on

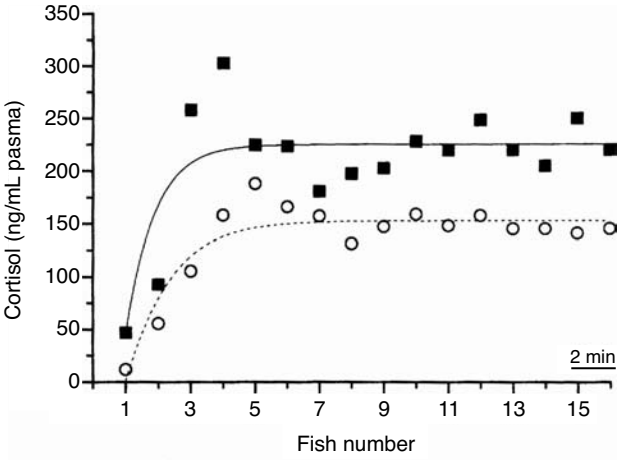

FIGURE 8.5 Effect of capture stress on plasma cortisol levels of Mozambique tilapia (*Oreochromis mossambicus*). Open circles are fish receiving food contaminated with PCB 126 (50 μg per kg fish per day for 5 days). The fish were captured subsequently at intervals of 2 minutes. Fish number 1 shows a resting cortisol level; the following fish already show a rise to a plateau level 5 minutes after. The plateau level of the PCB-treated fish is significantly lower than that of control fish (black squares), indicating an impaired cortisol response to capture by PCB 126. (From Quabius, E. S. et al., *Gen. Comp. Endocrinol.*, 108, 478–482, 1997. With permission.)

osmoregulation (McDonald and Milligan, 1997); however, the actual impact of the disruptive effects of some toxic chemicals on this process remains to be established.

The fact that toxic agents can act as stressors also implies that their effects are partially additive with those of other stressors. This is indicated by observations on Mozambique tilapia exposed to cadmium. In male fish with a low hierarchical position, the reduction of plasma osmolarity and the rise in plasma cortisol levels were more pronounced than in the dominant males (Wendelaar Bonga, 1997). Similarly, handling of the fish or crowding, known to cause a stress response in rainbow trout (Balm and Pottinger, 1995), makes these fish more sensitive to toxic agents. This is an aspect that requires more study not only in aquaculture, where, for example, the toxic effects of ammonia can be aggravated by poor holding conditions or rapid temperature changes, but also in toxicological risk analysis. For proper determination of no-effect levels or of lethal concentrations, any potentially stressful factor other than the toxic chemical should be absent. In many cases, the experimental conditions used to estimate toxicity did not meet this criterion. On the other hand, no effect and other toxicity levels determined under such rigorous conditions will easily lead to underestimation of the toxicity of a chemical in situations where the toxicant is not the only stressor.

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9

Toxic Responses of the Fish Nervous System

Steven P. Bradbury, Richard W. Carlson, Tala R. Henry, Stephanie Padilla, and John Cowden

CONTENTS

Introduction

Few of the approximately 70,000 chemicals on the Toxic Substances Control Act inventory or the 1000 to 1600 new chemicals introduced each year in the United States have been tested for neurotoxicity to support risk assessments (NRC, 1992), even though it is estimated that 5 to 10% of them are likely to be neurotoxic. Neurotoxicity has been defined as adverse effects of physical, biological, or chemical agents on the structure or function of the nervous system in developing or adult organisms (Philbert et al., 2000). From a human health risk assessment perspective, the potential for neurotoxic effects associated with synthetic chemicals has led to the development of valid, sensitive, and reproducible methods to identify neurotoxic chemicals, to characterize neurological effects, and to determine the mechanisms by which chemicals produce neurotoxicity. Similar efforts to develop methods for assessing neurotoxicity in fish may yield further insights into neurotoxic mechanisms (Carlson et al., 1998; Drummond and Russom, 1990; Featherstone et al., 1991, 1993; Rice et al., 1997; Timme-Laragy et al., 2006; Weber et al., 1997) in addition to addressing ecological risk concerns.

This chapter aims to provide a framework from which to approach questions concerning the neurotoxic effects of chemicals in fish. First, a brief summary of structural and functional attributes of the nervous system is provided, followed by an overview of neurotoxic mechanisms of action. The final section of the chapter summarizes mechanisms of action and manifestations of neurotoxic effects for several classes of compounds. In this latter section, an attempt is made to highlight examples where structural and functional alterations to the nervous system at the subcellular to cellular level can be linked to physiological and behavioral effects. The integration of effects across levels of biological organization is essential for establishing the mechanistic basis underlying neurotoxicity, as well as for identifying and quantifying ecologically relevant neurotoxic effects in fish.

Overview of Fish Nervous System Development, Structure, and Function

Development of the Fish Nervous System

The development of the fish nervous system follows the same general design as the development of all other vertebrate nervous systems (Figure 9.1). Beginning with gastrulation, in a process known as neural induction, ectoderm is specified into either surface epidermis or neuroectoderm. Surface epidermis ultimately forms skin, while the neuroectoderm becomes neural tissue. Evidence from several model organisms, including the widely used developmental model, zebrafish (*Danio rerio*), indicates that neural induction is mediated through the bone morphogenetic protein (BMP) signaling pathway. Surface epidermis is induced when ectoderm is exposed to BMP signaling. Extracellular antagonism of BMP ligands prevents BMP signaling, allowing the ectoderm to adopt a neuroectodermal fate (Blader and Strähle, 2000; Lewis and Eisen, 2003).

Following induction, the neuroectoderm undergoes several morphogenic movements to form the rudimentary nervous system (Lowery and Sive, 2004). Initially, the neuroectoderm forms a flat epithelial sheet called the neural plate (Figure 9.1A). As morphogenesis begins, the lateral edges of the neural plate rise to form the neural folds (Figure 9.1B). Specialized neural crest cells are induced at the apex of each neural fold. These neural crest cells migrate from the neural folds and ultimately give rise to several structures, including neurons and glia of the peripheral nervous system (PNS). The neural folds are brought into apposition and ultimately join at the midline. Unlike other vertebrates, fish neural fold fusion produces a solid rod of cells called the neural keel (Figure 9.1C). Although the midline is distinct during neural keel formation, cells within the neural keel are capable of crossing the midline. Once covered by surface epidermis, the neural keel subsequently undergoes programmed cell death to become

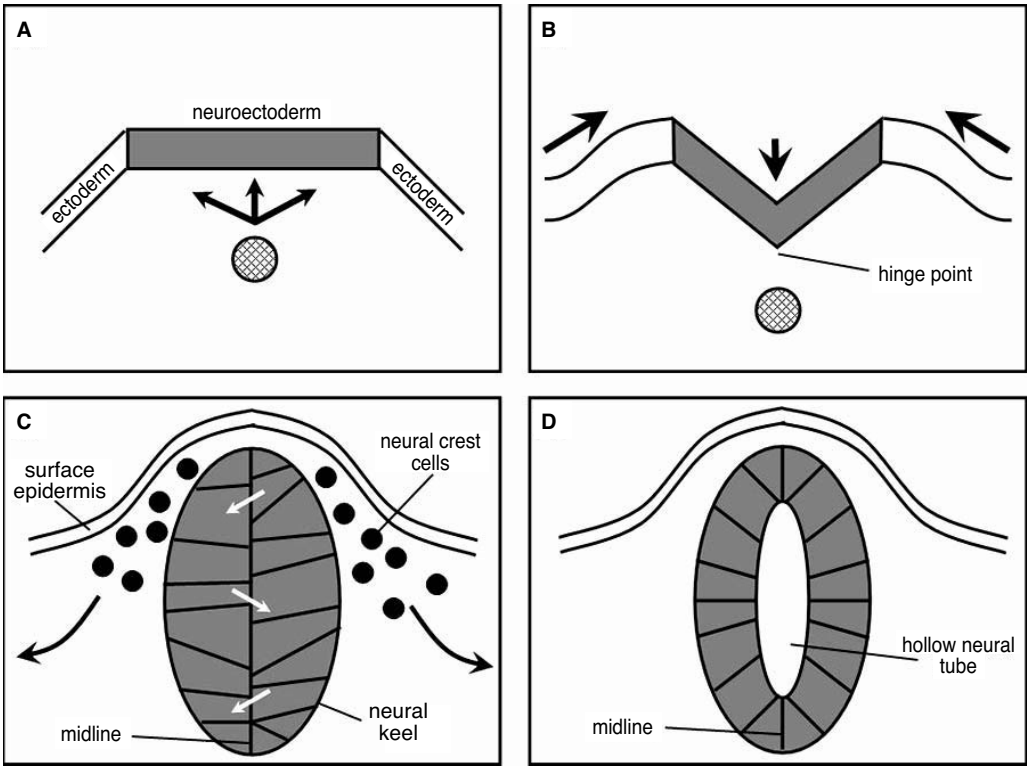

FIGURE 9.1 Overview of neural tube development. (A) BMP signaling antagonism allows the neuroectoderm to form from ectoderm, forming the neural plate. Signals from the notochord begin patterning the neural plate. (B) Morphogeneic movements begin forming the neural keel. (C) The neural keel is a solid rod of neuroectoderm tissue. Neural crest cells have begun migrating from the dorsal regions, and surface epidermis covers the neural keel. Unlike most vertebrates, cells within the neural keel are capable of crossing the midline. (D) Apoptosis of cells within the neural keel forms the hollow neural tube.

a hollow neural tube (Figure 9.1D). At the anterior end of the neural tube, bulging begins the subdivision of the brain into the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain) (Figure 9.2). The remainder of the neural tube becomes the spinal cord. Within the spinal cord, progenitor cells are patterned by secreted signaling molecules along the anterior–posterior and dorsal–ventral axes. Anterior–posterior patterning of the neuroectoderm begins during gastrulation in response to signals derived from the organizer (Blader and Strähle, 2000). Forebrain is thought to be the default state of neural tissue, with subsequent posteriorization involving retinoic acid, fibroblast growth factor, and Wnt signaling (Lewis and Eisen, 2003). Along the dorsal–ventral axis, distinct neuronal cell types are located at stereotypic positions in the spinal cord, with sensory neurons specified dorsally and motorneurons specified ventrally (Blader and Strähle, 2000; Lewis and Eisen, 2003). As with other vertebrates, sonic hedgehog protein secreted from the floor plate and notochord induces ventral motor-neuron differentiation in a concentration-dependent manner (Blader and Strähle, 2000; Lewis and Eisen, 2003). In the dorsal neural tube, BMP signaling from the roof plate and adjacent non-neural ectoderm are hypothesized to induce sensory neuron fates in a concentration-dependent manner (Blader and Strähle, 2000; Lewis and Eisen, 2003).

Once neuronal fates have been specified in appropriate anterior–posterior and dorsal–ventral locations, the next step in nervous system development is axonogenesis, when neurons extend axons to their innervation targets. Embryonic nervous system development has been particularly well characterized in zebrafish (*Danio rerio*) (Bernhardt, 1999); therefore, the summary of axonogenesis given here is necessarily restricted to zebrafish, although many facets of zebrafish axonogenesis are likely to be applicable

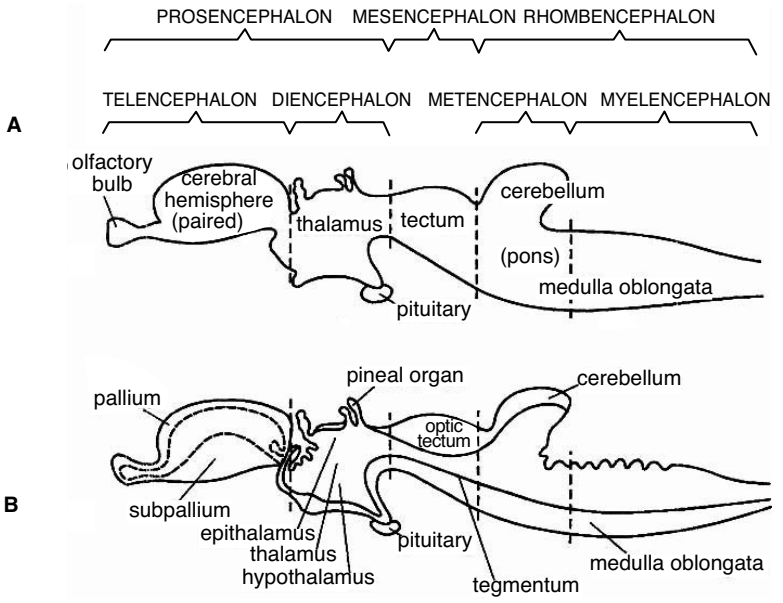

FIGURE 9.2 Major anatomical features of the fish brain (B is median section of A). (Adapted from Bond, C.E., *Biology of Fishes*, Saunders College Publishing, Orlando, FL, 1996, pp. 241–258.)

to other teleosts. A tremendous amount of descriptive information is known about zebrafish axonogenesis, with axonal pathways and targets characterized for several neuron classes (Bernhardt, 1999; Hutson and Chien, 2002; Lewis and Eisen, 2003). Unfortunately, the cellular and molecular basis for axon guidance is not as well understood. Recent genetic screens in zebrafish have identified mutants with defective axonogenesis (Hutson and Chien, 2002) and several axonal guidance gene homologs have been identified in the zebrafish genome (Bernhardt, 1999; Chilton, 2006). Although the presence of these homologs suggest that fish axon migration is regulated by integrating signals in the growth cone, the *in vivo* significance of these homologs remains to be determined.

The final step in embryonic nervous system development occurs when an axon reaches its innervation target. Active synapses are formed between neurons and their targets in a process known as synaptogenesis. Synaptogenesis has been heavily studied in higher vertebrates, with a generally accepted model of presynaptic neurons responding to both diffusible factors and distinct cell adhesion molecules in the target cell (Waites et al., 2005). Genes that play critical roles in vertebrate synaptogenesis are conserved in zebrafish (*Danio rerio*), although little functional assessment has been made (Hutson and Chien, 2002). Further insights into teleost synaptogenesis will likely come from real-time *in vivo* imaging of synaptogenesis in zebrafish (Hutson and Chien, 2002).

In the adult fish, the central nervous system (CNS) includes the neuronal structures encased within the skull and the spinal column. The PNS is comprised of nerve ganglia lying outside the spinal column as well as nerve processes found elsewhere throughout the organism. As noted above, this subdivision of the nervous system occurs during embryogenesis through ectodermal morphogenesis and patterning. The coordination and control of physiological and behavioral processes reflects the integration of structural and functional attributes of the entire nervous system.

Central Nervous System Anatomy

Although significant differences exist in anatomical features across fish species, the basic structural components of the developing CNS include the prosencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain), and the spinal cord (Figure 9.2).

Prosencephalon

The prosencephalon is comprised of the telencephalon and the diencephalon (Bond, 1996; Wullimann, 1998). The telencephalon possesses rostrally located olfactory bulbs, olfactory lobes, and, more caudally, cerebral hemispheres. The telencephalon is the site where the olfactory nerve (cranial nerve I) enters the olfactory bulbs. In actinopterygian fish, the cerebral hemispheres can be divided into dorsal and ventral components, which are homologous to the pallium and subpallium, respectively, of other vertebrates. The majority of afferent neurons entering the telencephalon are olfactory; however, there is some indication that non-olfactory ascending inputs exist, although their sites of integration have not been identified. It is now generally accepted that the telencephalon contributes to the processing of visual inputs, in addition to olfactory information, and plays a role in feeding, defense, schooling, aggressive, and reproductive behaviors (Bernstein, 1970; Bond, 1996; Davis and Kassel, 1983; Northcutt and Davis, 1983; Wullimann, 1998). Numerous studies have documented the presence of a variety of neurotransmitters in the telencephalon, as well as acetylcholine, gamma-aminobutyrate (GABA), catecholamines, excitatory amino acids (e.g., glutamate), and nitric oxide (Bissoli et al., 1989; Brantley and Bass, 1988; Byrd and Brunjes, 1995; Khan et al., 1996; Parent, 1983; Reiner and Northcutt, 1992; Sas et al., 1990; Schober et al., 1994; Sloley and Rehnberg, 1988; Sloley et al., 1992; Smith 1984). Catecholamines that have been identified in fish include noradrenaline, dopamine, and 5-hydroxytryptamine. Neuropeptides, including substance P, neuropeptide Y, and leucine-enkephalin (Byrd and Brunjes, 1995; Reiner and Northcutt, 1992), have also been found in fish nervous tissue.

The diencephalon in actinopterygian fish has been subdivided into the preoptic area, hypothalamus, thalamus, epithalamus, and synencephalon (Bradford and Northcutt, 1983). Wullimann (1998), however, considers the preoptic area an intermediate zone between the telencephalon and the diencephalon in the teleost brain. Wullimann (1998) also considers the synencephalon as part of the pretectum (see below), which is intermingled with diencephalic cell groups. Below the hypothalamus lies the pituitary gland, and the pineal organ arises from the epithalamus. The primary afferent neurons to the thalamus are the optic nerves (cranial nerve II), which enter the brain and cross anterior to the diencephalon. The hypothalamus receives a number of afferents from the telencephalon; the thalamus receives afferents from the cerebellum and serves as a relay center for the transfer of olfactory and striate-body impulses to other parts of the diencephalon and lower brain centers. In general, the diencephalon appears to serve as a coordination center for incoming and outgoing signals associated with internal homeostasis and for affecting the endocrine system through the pituitary gland (Bernstein, 1970; Bond, 1996; Bradford and Northcutt, 1983; Wullimann, 1998). Acetylcholine (Brantley and Bass, 1988), GABA, glutamate (Sloley et al., 1992), nitric oxide (Holmqvist and Ekstrom, 1997), noradrenaline, dopamine, and 5-hydroxytryptamine (Khan et al., 1996; Parent, 1983; Sas et al., 1990) have been identified as neurotransmitters in the diencephalon, primarily in the hypothalamus.

Mesencephalon

The mesencephalon, comprised of the optic tectum and the ventral tegmentum, is the primary site for processing impulses received from the optic nerve. Extensive electrophysiological and evoked response studies have been undertaken to create maps of visual fields on the optic tectum in cyclostomes, plagiostomes, and actinopterygians. The optic nerve is decussated, with projections from the right eye projecting to the left tectal hemisphere and from the left eye projecting to the right tectal hemisphere. Ascending efferent fibers from the optic tectum have been traced to the thalamus and pretectum, while descending efferent fibers proceed to the dorsolateral region of the ventral tegmentum. Some tectal outputs provide terminals to the reticular formation and cell groups in the medulla. Electrical stimulation and lesion studies support the conclusion that the optic tectum contains neuronal units that coordinate visual inputs with locomotor activity (Bernstein, 1970; Bond, 1996; Vanegas, 1983; Wullimann, 1998). Reports suggest that acetylcholine (Brantley and Bass, 1988), GABA (Rio et al., 1996), glutamate (van Deusen and Meyer, 1990), nitric oxide (Holmqvist and Ekstrom, 1997), and glycine (Becker et al., 1991) are predominate neurotransmitters within the optic tectum. Moderate catecholaminergic innervation has also been documented (Sas et al., 1990).

Rhombenchephalon

The rhombencephalon is comprised of the metencephalon and the myelencephalon. The metencephalon includes the cerebellum and the pons, with the cerebellum being the major component. The structure of the cerebellum is highly variable in fish. In teleosts, it can be divided into three major sections that are arranged similarly to that of the cerebellar cortex in other vertebrates. These areas are termed the valvula cerebelli, corpus cerebelli, and vestibulolateral lobe. Each area contains a molecular layer, a Purkinje (ganglion) cell layer, and a granule cell layer. Primary sensory fibers in hair cell sensory systems (see sensory organ systems discussion below) and neurons from the spinal cord are important ascending inputs to the cerebellum. Efferents from the cerebellum project to the ventral tegmentum, the thalamus, and the reticular formation. The cerebellum is associated with muscle tone, motor control, and lateral line sensory input (Bernstein, 1970; Bond, 1996; Finger, 1983; Wullimann, 1998). The cerebellum has been reported to contain glutamate, aspartate, GABA, and glycine, as well as catecholaminergic neurotransmitters (Ma, 1994; Sas et al., 1990). The myelencephalon, although containing portions of the cerebellum, is predominately comprised of the medulla oblongata (Wullimann, 1998). The boundary between the medulla oblongata and spinal cord in fish is generally indistinct and can be most readily defined by the types of information transmitted with associated nerve columns. Information derived from general sensory, cutaneous, vestibular, lateral line, and trigeminal (cranial nerve V) nerve fibers is carried in the somatic sensory column. Nerve fibers derived from chemoreceptors and nerves arising in the viscera are associated with the visceral sensory column. Sensory inputs associated with sight and olfaction do not input directly to the medulla. A visceral motor column in the medulla carries efferent fibers derived from the facial (cranial nerve VII), glossopharyngeal (cranial nerve IX), and vagus (cranial nerve X) nerves to the glands and musculature of the viscera. A somatic nerve column carries efferent nerve fibers to the ocular muscles and muscles of the pharyngeal complex. The medulla of the actinopterygians contains a pair of Mauthner cells, which are giant interneurons at the level of the vestibulocochlear nerve (cranial nerve VIII) that coordinate the startle response associated with sensory inputs. The dendrites of the Mauthner cells connect with fibers of the trigeminal nerve, facial nerve, glossopharyngeal nerve, and vagus nerve to the cerebellum and the optic tectum. Their axons pass the length of the spinal cord and coordinate musculature in the tail and associated rapid swimming movements (Bernstein, 1970; Bond, 1996). Serotonin and catecholamines have been reported to be neurotransmitters in the medulla (Parent, 1983; Sas et al., 1990), in addition to glycine, GABA (Becker et al., 1991; Faber and Korn, 1988; Legendre and Korn, 1994; Triller et al., 1993), and nitric oxide (Schober et al., 1994).

Spinal Cord

In fish, the spinal cord occupies the entire vertebral canal. The cord consists of a series of segments that form dorsal and ventral roots. The dorsal and ventral horns correspond to regions of sensory input (dorsal root) and motor output (ventral root). As is generally noted within vertebrates, these roots join to form the spinal nerves. Differentiation of the spinal cord increases with evolutionary progression from Cyclostomata (lampreys and hagfish) to Chondrichthyes (cartilaginous fish) to Osteichthyes (bony fish). In the latter fishes, the gray matter is clearly divided into dorsal and ventral horns. The dorsomedial gray matter innervates the trunk musculature and specialized areas, such as the pectoral fin. Many fibers ascend to the medulla oblongata and cerebellum. The descending fibers consist of many vestibulospinal and reticulospinal fibers and the giant Mauthner cells, which contain large-diameter, well-myelinated axons. The dendrites and cell bodies of Mauthner cells are found in the medulla oblongata. These cells have many collaterals that have extensive connections with interneurons and motorneurons in the spinal cord. The role of the Mauthner cells is to mediate sensory inputs through the startle response, as mentioned previously. As a final note, spinal cords of adult and larval fish are unique from mammals in their capacity for anatomical and physiological regeneration (Bernstein, 1970; Bond, 1996).

Peripheral Nervous System Anatomy and Function: The Autonomic Nervous System

The discussion of the central nervous system and associated components in the peripheral nervous system has made reference to aspects of both the somatic and autonomic nervous system, which are responsible for "voluntary" and "involuntary" actions. In mammals, the autonomic system contributes to the regulation

of organs and tissues such as the gut, heart, and blood vessels. In fish, the autonomic nervous system is similarly involved in the control of the gut and circulatory systems, as well as control of the swim bladder and melanophores. Studies in teleosts suggest that functional attributes of the autonomic nervous system are similar to that noted in mammals; however, the presence of gills and a swim bladder in fish and a smaller number of nerves and neurotransmitters highlight some important differences (Donald, 1998; Nilsson and Holmgren, 1998).

An anatomical differentiation of the fish autonomic nervous system into sympathetic and parasympathetic subsystems is difficult (reviewed in Donald, 1998; Nilsson and Holmgren, 1998). Instead, a terminology that differentiates cranial nervous pathways from spinal nervous pathways has been proposed. In both the cranial and spinal pathways, a preganglionic neuron runs from the central nervous system to an autonomic ganglion. A postganglionic neuron then serves as the afferent to the appropriate tissue or organ. The spinal autonomic ganglia are typically located close to the spinal column in higher vertebrates and teleosts, while autonomic ganglia associated with cranial pathways are usually located near or within the effector organ. Within the fishes, there is great variability in the anatomy of the autonomic nervous system. In the cyclostomes, the autonomic nervous system is poorly developed, with no spinal ganglia readily apparent, and it is difficult to differentiate sensory and autonomic nerve fibers. In the elasmobranchs and teleosts, autonomic ganglia are observed along the spinal column. Cranial autonomic fibers have been associated with the oculomotor (cranial nerve III), facial (cranial nerve VII), glossopharyngeal (cranial nerve IX), and vagus (cranial nerve X) nerves in the elasmobranchs. The teleost autonomic system most closely resembles that of higher vertebrates. Of note are the spinal ganglia that enter the cranium and have connections with the trigeminal (cranial nerve V)/facial (cranial nerve VII), glossopharyngeal (cranial nerve IX), and vagus (cranial nerve X) nerves. It is thought that innervation of the head and gills is associated with spinal ganglia. In the posterior region of the abdomen, the sympathetic chains fuse, and the vesicular nerves leave the chain to innervate the gut and urinogenital organs. Cranial autonomic fibers have been reported in the oculomotor (cranial nerve III) and vagus nerves (cranial nerve X), both of which reach the gut and swim bladder.

Although the number and nature of neurotransmitters are not as well resolved in fish as in mammals, compounds representative of those seen in higher vertebrates have been characterized. Assessments of catecholamine levels indicate that both adrenaline and noradrenaline are likely stored and released in adrenergic synapses. It is generally assumed within the vertebrates that acetylcholine is found in all postganglionic, parasympathetic neurons. In some teleosts, evidence for the presence of acetylcholine has been reported for the spleen, heart, and swim bladder (Abrahamsson et al., 1979; Balashov et al., 1981; Holmgren, 1977; Hsieh and Liao, 2002; Ishimatsu et al., 1986; Ovais et al., 1976; Thompson and O'Shea, 1997). Neurons containing 5-hydroxytryptamine which innervate the gut of several cyclostome and teleost species have been reported (Anderson, 1983; Anderson and Campbell, 1988; Anderson et al., 1991; Watson, 1979). Vagal neurons that innervate the gills in rainbow trout (*Oncorhynchus mykiss*) have also been reported to contain 5-hydroxytryptamine (Saltys et al., 2006; Sundin, 1995; Sundin et al., 1998). Using immunohistochemistry and radioimmunoassay techniques, a number of suspected neuropeptides have also been identified; for example, bombesin has been identified in autonomic nerves of the gut and circulatory system in several fish species (Bjenning and Holmgren, 1988; Bjenning et al., 1990; Cimini et al., 1985; Holmgren and Nilsson, 1983; Langer et al., 1979). In addition, neuropeptide Y, vasoactive intestinal polypeptide, and several somatostatins and tachykinins have been identified in fish (Donald, 1998; Nilsson and Holmgren, 1998). Nitric oxide has also been reported as a putative neurotransmitter in the autonomic nervous system (Donald, 1998).

Sensory Organ Systems

The sensory systems of fish can be divided into vision, auditory, mechanosensory, electrosensory, and chemoreception. In the assessment of neurotoxicity, these sensory organ systems are viewed as readouts of a functional nervous system. The eyes of fish generally have the same structures as those noted in the vertebrates, including the anterior chamber, an iris, a lens, and a vitreous chamber that is lined by the retina. The retina is comprised of pigmented epithelium, which consists of photoreceptors (rod and cone cells), horizontal cells, bipolar cells, amacrine cells, and ganglion cells and nerve fibers that lead

to the optic nerve. The optic nerve is comprised of four different types of fibers, most of which are afferent and project to the contralateral side of the brain (the optic tectum). Horizontal cells facilitate information flow between photoreceptors, and amacrine cells facilitate lateral flow of information among ganglion cells. Bipolar cells direct excitatory or inhibitory responses vertically to the ganglion cells, which subsequently send information to the CNS through the optic nerve. A variety of rod and cone subtypes are observed in fish and suggest specialization in terms of discriminating wavelengths and intensity of light (Hawryshyn, 1998). Freshwater fish are generally found to have porphyropsin as their main photosensitive pigment, while marine fish typically have rhodopsin in their retina (Bond, 1996). As reviewed by Hawryshyn (1998) and Lasater (1990), glutamate is the putative neurotransmitter in the photoreceptors that mediates synaptic communication with bipolar cells, while GABA has been well-documented in horizontal and amacrine cells (reviewed in Hawryshyn, 1998; Lasater, 1990; Marc and Cameron, 2001). Glycine, dopamine, and acetylcholine have also been reported as neurotransmitters in the retina. A potential role of neuropeptides has yet to be elucidated.

The auditory, mechanosensory, and electrosensory systems include the inner ear, the lateral line (comprised of the neuromasts and canals), and the ampullary and tuberous organs of the electrosensory lateral line (Bond, 1996; Schellart and Wubbels, 1998). The ear functions contribute to balance and sound reception, while lateral line functions are related to sensing water displacement and pressure. The electrosensory lateral line is responsible for sensing electrical fields and signals. The inner ear of fish includes three otolith organs: the saccule, lagena, and utricle organs. The sacculus and lagena are primarily responsible for sound reception. The sensory epithelium, termed the macula, is comprised of sensory hair cells that are the receptor cells. Auditory stimulus causes deflections of hair bundles, leading to a depolarization of hair cells and subsequent increase in the firing rate of afferent nerve fibers to the brain. The auditory nerve (vestibulocochlear nerve; cranial nerve VIII) innervates the macula. Those portions of the brain thought to integrate auditory inputs have been discussed previously. The mechano-sensitive lateral line consists of neuromasts that are found over the entire body, either free on the skin or within pits, grooves, or canals. A neuromast consists of sensory hair cells with supporting cells, all of which are covered by a cupula. Some neuromasts are open to the water, via pores in the bone or scales. Water movement causes an impulse of fluid within the lateral line that in turn causes a deformation of the cupulae. This deformation of the hair cells results in a response in the nerve fiber innervating the neuromast. The number of innervating nerve fibers can range from a few to several hundred. The dorsal anterior lateral line nerve innervates the dorsal part of the head, the ventral lateral line nerve innervates the cheek and lower jaw, and the posterior lateral line nerve innervates the trunk and tail. The involvement of different portions of the brain in integrating responses to these inputs has been discussed previously. Of note are the projections of auditory and lateral line afferents to the Mauthner cells, which invoke the startle response.

Chemosensory systems in fishes are primarily divided into gustation and olfaction and are well developed in fish. Taste buds are comprised of 100 to 150 specialized epithelium cells, which include receptor cells, basal cells, and supporting cells. Taste buds are found primarily in the mouth, pharyngeal region, and gill arches; however, in some species they may be found on barbels, fins, and other parts of the body surface. Taste buds in the oropharyngeal cavity are innervated by the glossopharyngeal (cranial nerve IX) and vagus (cranial nerve X) nerves, while the facial nerve (cranial nerve VII) innervates taste buds on external surfaces. Receptor cells within the taste buds respond to specific compounds, such as amino acids. Many taste receptors are thought to be ion channels or proteins associated with ion channels. Binding of chemicals to the appropriate receptor elicits depolarization of the receptor cell. Through chemical synapses, the potential of the innervating nerve fiber may be modulated prior to propagation to the CNS (Bond, 1996; Sorenson and Caprio, 1998). The olfactory organs are found in pits on the anterior portion of the head. The epithelium within the olfactory chambers is arranged in a series of lamellae that form the olfactory rosette. Both sensory and nonsensory cells are found in the epithelium. The nonsensory cells serve to protect the sensory cells and transport chemical stimuli. Ciliated and microvillous receptor cells have been noted in fish. These cells are innervated by the olfactory nerve, which provides input to the olfactory bulb. The receptor cells are responsive to amino acids, bile acids, gonadal steroids, and prostaglandins (Byrd and Caprio, 1982; Caprio and Byrd, 1984; Chang and Caprio, 1996; Ohno et al., 1984; Sato and Suzuki, 2001). A variety of alcohols, amines, carboxylic acids, nucleotides, and hydrocarbons

can also elicit olfactory responses (Kolesnikov and Kosolapov, 1993; Rolen et al., 2003). Specific receptor proteins in sensitive cells are thought to be responsible for initial olfactory responses. These receptors initiate second-messenger-mediated signal transduction pathways that open ion channels and lead to the production of action potentials (Bond, 1996; Sorenson and Caprio, 1998).

Basic Mechanisms of Neurotoxicity

Before considering some mechanisms of neurotoxicity in adult fish, it is important to remember that neurotoxic compounds are also likely to have developmental effects. In particular, the potency and mode of action of neurotoxic compounds may be dramatically altered during maturation of the nervous system. To underscore the importance of this concept, compounds with adverse effects on the immature nervous system are categorized as developmental neurotoxicants.

It is widely accepted that the developing nervous system is particularly susceptible to some neurotoxic compounds (Costa et al., 2004; Mendola et al., 2002; Stein et al., 2002). This assertion is based largely on the fact that neurotoxicant exposures having little to no effect on the mature nervous system may produce significant and lasting effects on the developing nervous system. Several processes critical for proper nervous system structure and function, including neuronal specification, migration, and synaptogenesis, all occur *in ovo*. Furthermore, several neurotransmitters have developmental roles that are distinct from their synaptic roles (e.g., acetylcholine) (Lauder and Schambra, 1999). Disruptions in any of these processes or neurotransmitters may have unique effects during development that are not seen in the mature nervous system and are potential mechanisms of developmental neurotoxicity.

In the mature nervous system, neurotoxic mechanisms of action can be associated with a variety of unique anatomical and physiological characteristics of the nervous system (Anthony et al., 1996; Philbert et al., 2000). Many aspects of the mammalian nervous system contribute to its vulnerability: maintenance of the blood–brain or blood–nerve barrier, high energy requirements, high lipid environment, complex spatial geometry, and the need for rapid transmittal of information between cells. Because the structural and functional aspects of neurons are highly conserved, the framework proposed by Anthony and coworkers (1996) for understanding mechanisms of neurotoxicity, as well as the manifestations of toxic effects, is relevant and briefly reviewed.

In terms of xenobiotic exposure, the central and peripheral nervous system are unique in that tissue and cellular attributes minimize the transport of potentially toxic compounds to sites of action. The restricted interfaces between the blood and many components of the nervous system have been termed as barriers: the blood–brain barrier encloses the central nervous system, and the blood–nerve barrier encloses the peripheral nervous system. The basis for the blood–brain barrier is that the endothelial cells lining the vasculature of the brain have extremely tight intercellular junctions. As a consequence, to gain access, xenobiotics are required to diffuse through these cellular barriers. The lipid-rich myelin surrounding axons, which is critical for insulating the nerve and ensuring rapid propagation of action potentials, may provide a barrier to hydrophilic xenobiotics. In general, the more hydrophilic the compound, the less likely it will cross one of these barriers; however, some hydrophilic compounds may gain access by piggybacking on specific transporters that are present in these barriers. Oligodendrocytes and Schwann cells found in the central and peripheral nervous systems, respectively, are responsible for maintaining the myelin sheaths that exclude water and ions from the axons and for minimizing extracellular spaces. A wide variety of specialized proteins must be synthesized and complex lipid metabolism must be maintained to support the myelin environment. Neurons also need to maintain ion gradients to support neuronal transmission. These maintenance activities require a high aerobic metabolic rate. As a consequence, the nervous system requires high levels of oxygen and glucose to maintain homeostatic ion gradients and to restore ion gradients after depolarizations.

Finally, the unique spatial arrangement of the nervous system makes it vulnerable to toxic insults. The nervous system is comprised of relatively large cells as the result of evolutionary pressures to establish high-speed intracellular communication; consequently, axon volumes are typically much greater than those of the cell bodies and have a unique demand for macromolecule synthesis. The cell body of each neuron synthesizes everything that is needed by the rest of the cell (axons and dendrites); these

products are then transported throughout the cell to replenish cellular constituents. In addition, spent cellular constituents are transported retrogradely from the processes to the cell body. The retrograde transport also contains biochemical information related to the status of the environment in the distal portion of the neuron. Spatial arrangement of the nervous system also requires maintenance of the transmission of electrical pulses along the length of axons and across the synapses between neurons. Maintenance of both intracellular and extracellular communication is, of course, essential to the proper function of the nervous system.

In conclusion, the nervous system has a variety of unique structural characteristics that meet the need for rapid cellular communication. These unique structural characteristics are primarily associated with maintaining the integrity of the cell body and axon and the ability of the nerves to support propagation of action potentials and synaptic transmission. Xenobiotics capable of disrupting ion channels that are essential to maintaining and supporting proper ion balances or capable of disrupting chemical transmission of potentials across synapses are capable of causing neurotoxic effects. As discussed previously, the maintenance of aerobic respiration, high rates of protein and lipid synthesis, and extensive transport of synthetic products from the cell body to the axon are critical for maintaining structural and functional characteristics of the nervous system. Consequently, xenobiotics capable of disrupting neuron-specific synthetic and metabolic pathways, modifying the products of these reactions, or inhibiting or uncoupling aerobic metabolism can elicit adverse effects specific to the nervous system. It is interesting to note that some fish species have evolved specific metabolic strategies for anoxic conditions. Research suggests that anoxic-tolerant species may have decreased levels of excitatory neurotransmitters and increased levels of inhibitory transmitters in the brain that enable metabolic depression (Van Ginneken et al., 1996).

Manifestations of Neurotoxicity in Fish

Neurotoxic effects of chemicals are assessed by quantifying structural and functional responses at the subcellular to organismal levels of biological organization. Functional observations at the organismal and cellular levels can provide insights concerning potential sites and modes of action, while cellular and biochemical responses can provide insights on molecular mechanisms of action. Cellular and biochemical investigations can be used to identify neurotoxic potential, characterize the nature of neurological effects, and determine the mechanisms by which chemicals produce neurotoxic effects (Tilson, 1996). A significant challenge in developing investigative methods and associated bioassay techniques lies in linking neuromorphological, neurochemical, and neurophysiological alterations with functional (i.e., behavioral) observations (NRC, 1992). Many chemically induced biochemical, physiological, or morphological perturbations have been reported in cellular and organismal systems, but consequent behavioral effects on organism have not been established. Because behavioral responses are an integration of biochemical, physiological, and morphological processes, linking behavioral observations to these types of observations can provide the needed bridge between subcellular and cellular responses and ecological consequences (Little, 1990). Examples where chemically induced biochemical, physiological, or morphological perturbations have been mechanistically linked to ecologically relevant behavioral responses in fish are limited.

Structural Manifestations of Neurotoxicity in Fish

As discussed previously, dynamic interactions between the neuronal cell body and the axon are critical to maintaining proper neuronal structure and function. Disruption of these interactions can result in a variety of pathologies. Neuronopathies result from toxicants capable of causing injury to the cell body followed by degeneration of the remaining cell processes. Neuronal loss is typically permanent and is manifested by global symptoms or dysfunctions consistent with the specific nervous tissue target; for example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes neuronopathies in mammals through its cytotoxic dihydropyridium ion metabolite. Similar observations have been reported in fish

(Poli et al., 1990; Pollard et al., 1992). Axonopathies are associated with toxicants whose primary site of action is the axon, causing degeneration of the axon along with surrounding myelin. Because the nerve cell body is not affected, recovery and regeneration of the axon are possible. The effects of neurotoxicants capable of eliciting axonopathies are sometimes described as causing chemical transections of the nerve (e.g., acrylamide, carbon disulfide, some diketones, and certain classes of organophosphorus esters). Likewise, compounds disrupting microtubles (such as colchicine) will perturb axonal transport and thereby cause pathology. Myelinopathies are associated with compounds capable of causing intramyelinic edema, such as hexachlorophene (Kinoshita et al., 2000; Yoshikawa, 2001). Hexachlorophene-induced myelinopathies have also been reported in non-mammals (Reier et al., 1978). Other compounds are capable of causing the selective destruction of the myelin, resulting in neuronal demyelination. These responses may be due to effects on the myelin itself or due to effects on the myelinating cells.

Physiological Manifestations of Neurotoxicity in Fish

Electrophysiology

Electrophysiological techniques measure electrical potentials and the transmission of impulses in the nervous system. These techniques can be applied to *in vitro* or *in vivo* preparations and used to assess responses of the brain, spinal cord, components of the peripheral nervous system, or sensory systems to determine whether or not a neurotoxic response can be elicited by a xenobiotic. Electrophysiological techniques can be used to determine if a toxicant acts pre- or postsynaptically on specific portions of a fiber or on specific ion channels.

Measurements can be made from the surface of the organism, extracelluarly or intracellularly (Baker and Lowndes, 1986; Eisenbrandt et al., 1994; Fox et al., 1982). Bahr (1972, 1973), for example, reported an *in vivo* method to record evoked electrical activity of the trunk lateral line in surgically prepared rainbow trout (*Oncorhynchus mykiss*) for periods of up to 48 hours. Evans and Hara (1985) described an *in vivo* rainbow trout model to quantify evoked electro-olfactograms, and Kreft and coworkers (1985) described a technique to assess electroretinograms from photostimulated *in vitro* eye preparations. Schafer and coworkers (1995) described an *in vivo* anesthetized catfish (*Ameiurus nebulosus*) preparation to measure spontaneous afferent activity from electroreceptor organs. Several whole-cell and membrane preparations have also been used to study ion channels and the role of specific neurotransmitters in channel function; for example, voltage clamp analysis has been used to study the role of glycine, GABA, and *N*-methyl-D-aspartate (NMDA) in isolated lamprey spinal cord neuron function and to identify calcium channel subtypes in lamprey sensory and motorneurons (Baev et al., 1992; El Manira and Bussieres, 1997; Moore et al., 1987).

To date, most electrophysiological techniques applied to fish involved *in vitro* models or surgically invasive approaches use restrained or anesthetized animals. Although these methods yield data regarding cellular and molecular mechanisms of neurotoxic action, disruption of the nervous system or sensory organs can make it difficult to study behavioral outcomes of perturbed neurological function. Two laboratories (Carlson et al., 1998; Featherstone et al., 1991, 1993) have described an approach relating electrophysiological responses to a defined behavioral endpoint. In these studies, the electrophysiological responses of Mauthner cells, motorneurons and interneurons, and white musculature to an invoked startle response were measured *in vivo* in unanesthetized, freely moving larval fish. These waveforms are easily triggered and recorded, yielding stereotypical and reproducible results.

Respiratory–Cardiovascular

The effects of toxicants on the central or peripheral nervous system can elicit a wide variety of integrated respiratory–cardiovascular responses. In turn, these responses can be evaluated to determine the extent to which they can be associated with specific mechanisms of neurotoxic action. For example, several studies have demonstrated that specific suites of respiratory–cardiovascular responses of spinally transected rainbow trout (*Oncorhynchus mykiss*) were sufficient to differentiate effects associated with industrial organic chemicals whose sites and mechanisms of action are distinct, including narcotics,

acetylcholinesterase inhibitors, pyrethroid insecticides, cyclodiene insecticides, and strychnine (Bradbury et al., 1991a; Bradbury and Coats, 1989; Bradbury et al., 1991b; McKim et al., 1987). More specifically, distinct suites of effects on cough frequency, ventilation frequency, ventilation volume, oxygen consumption, and arterial blood oxygen, carbon dioxide, pH, hemoglobin, and hematocrit were associated with the different compound classes studied. Furthermore, these respiratory–cardiovascular responses were consistent with the neurodepressant, stimulant, or convulsant mechanisms of action of the xenobiotics studied.

Behavioral

The behavior of a fish reflects the integrated output of the nervous system at the organismal level in response to stimuli perceived in the environment. The extent to which these chemically induced behavioral changes are ecologically relevant must be considered in terms of adverse effects on an organism's ability to survive and reproduce. Behavioral endpoints can be categorized as individual or interindividual responses (Rand, 1985). Individual responses include undirected and directed locomotion and feeding. Undirected locomotion refers to the movement of an animal that is not related to the intentional placement of a stimulus. The direction of undirected locomotion is considered random. This spontaneous activity can be affected by neurotoxicants and described with a variety of qualitative descriptors (Heath, 1995). Although such observations may be difficult to interpret, Drummond and Russom (1990) demonstrated that a behavioral response checklist could be used to categorize toxicants with known modes of action within specific syndromes. Heath (1995) and Rand (1985) also summarized a variety of experimental approaches whereby fish locomotion responses can be quantitatively measured following exposure to a wide variety of organic and inorganic neurotoxicants; for example, undirected locomotion can be quantified by measuring water currents created by the movement of fish or by measuring voltage changes in the water caused by swimming fish. The use of photoelectric gates and video cameras to quantify swimming speed and exploratory activity has also been described.

Directed locomotion refers to movement in response to specific external stimuli. The ability of a fish to swim with or against a water current (negative and positive rheotaxis) is a commonly studied forced locomotive response. The directed movement of fish due to avoidance or attractiveness to xenobiotics or natural chemical stimuli has also been examined in some detail (reviewed in Hara et al., 1983; Heath, 1995). Chemosensory disruption can result in overt preference or avoidance to a xenobiotic. Avoidance of or preference to natural and xenobiotic chemical cues during or following xenobiotic exposure can also occur. Disruption in normal locomotion can be quantified by observing movements of an organism across chemical gradients. The locomotion of a fish in response to a xenobiotic is likely the result of interactions with the olfactory or gustatory receptors; however, avoidance can also be the result of irritation of mucous membranes. The ability of a xenobiotic to mask or counteract natural chemical signals used in migration, for example, may be the consequence of competition for receptor sites on sensory cell membranes. Alternatively, the xenobiotics may be directly toxic to receptor cells (Hara et al., 1983). As reviewed by Heath (1995), a wide variety of xenobiotics, including metals, petroleum constituents, detergents, and insecticides, have been observed to depress fish feeding rates. This depression may be associated with effects on olfactory, gustatory, visual, or lateral line receptors or effects on the ability of the central nervous system to integrate environmental stimuli.

Of the interindividual responses, territoriality, dominance, schooling, and predator–prey interactions have been studied most extensively (Heath, 1995; Rand, 1985). Predator–prey interactions have been examined in studies in which either the predator or the prey is exposed to the xenobiotic, as well as in studies where both sets of organisms are exposed. Endpoints in these studies can include prey survival rates, predation rates, or prey handling times. Although effects on predator–prey interactions have been observed for a variety of insecticides, herbicides, and metals, the mechanistic basis for these effects is largely unknown. The need to understand fully the basis of xenobiotic effects on predator–prey interactions has been well articulated by Sandheinrich and Atchison (1990). In this regard, Carlson and coworkers (1998) reported an attempt to relate the effects of a wide variety of organic xenobiotics on prey survival in terms of electrophysiological responses of the Mauthner-cell-mediated startle response, which initiates an escape behavior in response to predation.

Examples of Major Classes of Neurotoxicants

Narcotics

Research reported during the early 1980s in both the United States and The Netherlands established that the majority of industrial organic chemicals (excluding pesticides and pharmaceutical agents) elicit acute toxic effects in fish through a narcosis mechanism (Konemann, 1981; Veith et al., 1983). Narcosis can be defined as a *reversible* state of arrested activity of protoplasmic structures resulting from exposure to a xenobiotic. In the context of the intact organism, the terms *narcosis* and *general anesthesia* are commonly used interchangeably (Bradbury et al., 1989). Although typically described in the literature as a nonspecific mode of action, the actual mechanism of narcosis and anesthesia remains unknown and is an active area of research, as discussed in several reviews (Århem et al., 2003; Bradbury et al., 1989; Franks and Lieb, 1990, 1994; Franks and Lieb, 2004; Narahashi et al., 1998; Pryor, 1995).

Mechanisms of Narcotic Neurotoxicity

Both lipophilicity (Meyer–Overton rule) and thermodynamic activity (Ferguson's rule) (Ferguson, 1939) have been demonstrated to be related to the narcotic potency of chemicals. Thus, varying aqueous concentrations of different compounds may be required to cause comparable effects, even though their thermodynamic activity at the site of action is proposed to be the same. There is, however, ample evidence of narcotics and anesthetics whose potency is not consistent with thermodynamic theory. These departures from thermodynamic consistency suggest that sometimes potency must be viewed as a more complex toxicological and physicochemical interaction between the xenobiotic and a site of action. As a result, investigators developing hypotheses to explain the molecular events of anesthesia/narcosis are increasingly acknowledging that their models must accommodate the likelihood of multiple sites and mechanisms of action (Bradbury et al., 1989; Franks and Lieb, 1990, 1994; Franks and Lieb 2004; Miller 2002; Narahashi et al., 1998; Pryor 1995). Within the last 10 years, it has become increasing apparent that anesthesia-like compounds interact directly and specifically with proteins, most notably proteins that form ion channels (Århem et al., 2003; Franks and Lieb, 1990, 1994, 2004; Narahashi et al., 1998).

Franks and Lieb (1990, 1994, 2004) proposed that anesthesia is the result of direct interactions between xenobiotics and neuronal ion channels. This hypothesis evolved partly from x-ray diffraction experiments undertaken by these investigators showing insignificant changes in lipid membrane bilayers at physiologically relevant concentrations of narcotic xenobiotics (although see the study by Cantor, 1997). Subsequent studies with firefly luciferase established that inhibition of luciferase activity correlated with anesthetic potency and that the sites of action can have polar and nonpolar characteristics. These investigators also demonstrated that narcotics caused reversible inhibition of specific, spontaneously firing neurons in the giant snail. This inhibition was saturable and consistent with binding to a receptor site. While the specific proteins associated with narcosis have yet to be identified, an implication of this hypothesis is that classes of receptors, or receptor sites, with varying hydrophobic and hydrogen-bonding characteristics may be involved. Differential effects on target proteins, or proteins in different neuron classes, could provide an explanation for different narcosis/anesthetic effects observed at the cellular through organismal level (Bradbury et al., 1989).

In conclusion, hypotheses developed to date provide implicit or explicit basis for attributing narcosis to more than one site of action or mechanistic process. In general, all of the hypotheses attribute narcosis to neuronal dysfunction ultimately caused by changes in the properties of neuronal ion channels, which are beginning to be identified (reviewed in Århem et al., 2003).

Manifestations of Narcotic Neurotoxicity in Fish

With the development of initial acute toxicity datasets for industrial organic chemicals, it was established that the potency of narcotics in fish was dependent on the hydrophobicity of a xenobiotic (Konemann, 1981; Veith et al., 1983). Subsequent experimental studies and modeling efforts have led to general acceptance that the relationships between hydrophobicity and lethality represent the minimum, or

baseline, toxicity that a compound can elicit in the absence of a more specific mode of toxic action. With additional study it became clear that there are subclasses of narcotics, more potent than would be predicted from baseline narcosis, that could be classified on the basis of either acute potency or physiological and behavioral characteristics of the narcosis response. More specifically, narcosis induced by certain esters, phenols, and anilines (typically termed polar narcotics) seemed to be unique (Bradbury et al., 1989).

Behavioral and gross morphological signs of stress in fathead minnows (*Pimephales promelas*) associated with acutely lethal aqueous exposures to baseline narcotics include depressed locomotor activity with little or no response to outside stimuli. Body color also becomes darker as fish are increasingly intoxicated. Most fish die within 24 hours, but effects are reversible if fish are transferred to "clean" water prior to death. In contrast, acutely lethal concentrations of polar narcotics with log octanol–water partition coefficients below 2.7 elicit hyperactivity and usually overreaction to outside stimuli for 24 to 48 hours, with subsequent depression and death (Drummond and Russom, 1990). Medaka (*Oryzias latipes*) larvae exposed to phenol and 1-octanol at levels 2 to 3 times lower than acutely lethal concentrations were more susceptible to predation by bluegill (*Lepomis macrochirus*) than were unexposed fish (Carlson et al., 1998).

To evaluate further the symptomology of narcosis in fish, researchers (Bradbury et al., 1989; McKim et al., 1987) examined the respiratory–cardiovascular responses of spinally transected rainbow trout (*Oncorhynchus mykiss*) to baseline narcotics (1-octanol and MS-222) and polar narcotics (phenol, 2,4-dimethylphenol, aniline, 2-chloroaniline, and 4-chloroaniline). The responses of the trout exposed to these groups of compounds were distinct. The overall response to the baseline narcotics was a dramatic slowing of all respiratory–cardiovascular functions. While ventilation volume and oxygen consumption decreased, oxygen uptake efficiency increased as water flow over the gills slowed and the blood-to-water perfusion ratio increased. A rapid drop in heart rate (reflex bradycardia) was thought to be related to an increase in vagal tone caused by hypoxia. As respiration rate declined, total arterial blood oxygen and pH also decreased. The associated increase in hematocrit, caused by red blood cell swelling, is well documented during anesthesia and is associated with hypoxia. These effects are reversible, as demonstrated by experiments in which fish at the point of respiratory failure could be revived if clean water was perfused across the gills.

In general, the respiratory–cardiovascular symptoms associated with baseline, or nonpolar narcosis, are consistent with depressant anesthesia as described by Winters (1976). The most striking feature associated with exposure of rainbow trout (*Oncorhynchus mykiss*) to lethal aqueous concentrations of polar narcotics was the development of tremors and clonic seizures that were initiated by coughs (Bradbury et al., 1989). These tremors and seizures originated in the head and moved posteriorly to include the tail, even though the fish were spinally transected. These observations suggest that polar narcotics affect the spinal cord posterior to the transection, or perhaps the peripheral nervous system. In mammals, the primary site of phenol stimulation is thought to be the spinal cord (Deichmann and Keplinger, 1981). With increasing length of exposure, seizure intensity subsided, and the fish became unresponsive to outside stimuli. Consistent with the increased activity associated with seizures and muscular activity, oxygen uptake initially increased, yet ventilation volume and frequency eventually declined which is more consistent with a general depressant effect. Depressions of arterial blood oxygen, carbon dioxide, and pH and an associated increased hematocrit were consistent with a shift to anaerobic metabolism during seizures and subsequent respiratory failure. Fish could be revived by artificially irrigating the gills. The responses of fish to polar narcotic exposure are generally consistent with the description of cataleptic anesthesia (Winters, 1976).

Carlson and coworkers (1998) investigated the sublethal effects of 1-octanol and phenol on *in vivo* electrical impulses generated within the Mauthner cells and associated interneurons, motorneurons, and axial musculature during the startle response reflex in larval medaka (*Oryzias latipes*). With 1-octanol, electrical waveforms were depressed at exposure concentrations 5 times lower than the 48-hour LC_{50}, and the ratios of startle responses to stimuli were significantly depressed. These observations are suggestive of a sensory deficit due to an anesthetic-like effect. Phenol caused a significant decrease in the motorneuron-to-muscle delay, consistent with the initial hyperactivity and sensitivity noted in exposures to polar narcotics and reports that phenol stimulates the mammalian spinal cord (Deichmann

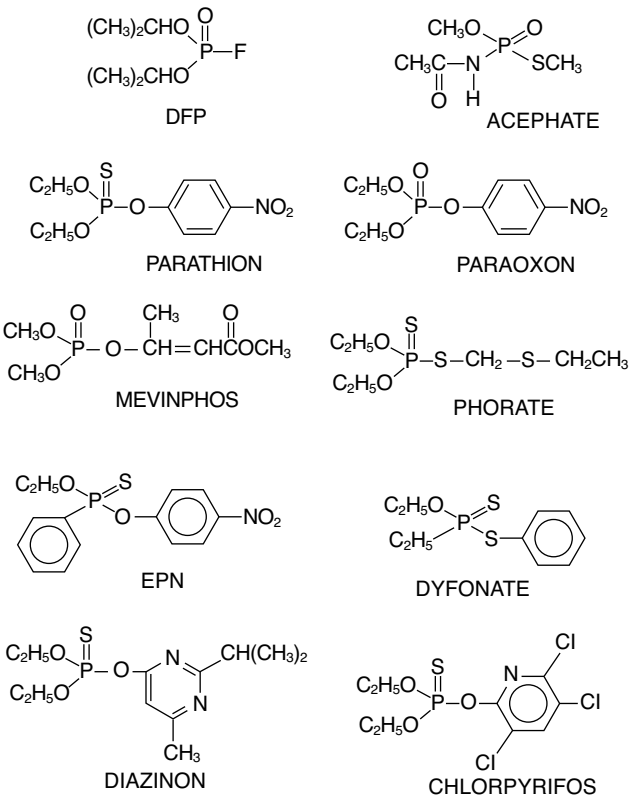

FIGURE 9.3 Examples of organophosphorus insecticides.

and Keplinger, 1981). These electrophysiological studies generally support other toxicodynamic and behavioral studies that suggest baseline and polar narcotics act through different mechanisms or sites of action.

Cholinesterase Inhibitors

Several neurotoxic compounds are known to target the cholinergic nervous system. By far the largest classes are the organophosphorus and carbamate compounds. Organophosphorus and carbamate compounds have been used extensively as insecticides, herbicides, and fungicides. Initial studies on the synthesis and activity of organophosphorus insecticides were undertaken in Germany during the 1930s. Agricultural use of insecticidal organophosphorus and carbamates increased in the 1960s as the use of organochlorine compounds declined. In the United States, use of organophosphorus insecticides peaked in the mid-1970s and has declined with increased use of pyrethroid and carbamate insecticides. Insecticidal carbamates were first introduced in 1954 and are still employed for agricultural and household applications (Abou-Donia, 1995; Metcalf, 1995).

Organophosphorus insecticides are organic phosphoric ester compounds with the general structure of $R_1P(=Y)(R_2)X$, where Y is an oxygen or thio group, X is a leaving group (e.g., halide, phenoxy, or other group), and R_1 and R_2 are typically alkyl, alkoxy, aryl, or aryloxy substituents. Representative structures of organophosphorus insecticide classes are depicted in Figure 9.3. Carbamate insecticides are organic compounds that contain a RNC(=O)O moiety; examples of common *N*-methylcarbamate and *N*-methylcarbamoyl oximes are provided in Figure 9.4. While organophosphorus and carbamate insecticides are structurally diverse, their common neurotoxic mechanism is related to their ability to phosphorylate or carbamylate esterases.

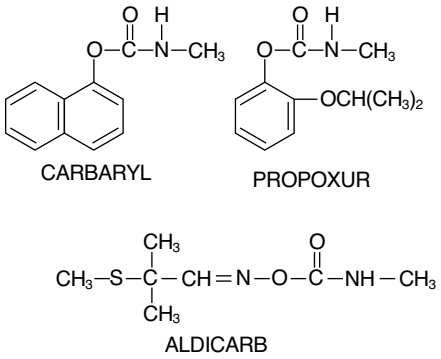

FIGURE 9.4 Examples of *N*-methylcarbamate (carbaryl and propoxur) and *N*-methylcarbamoyl oxime (aldicarb) carbamate insecticides.

Mechanisms of Cholinesterase Inhibitor Neurotoxicity

The neurotoxic mechanism for organophosphorus and carbamate insecticides is inhibition of acetylcholinesterase (AChE) in the CNS and PNS (Figure 9.5) (reviewed in Ecobichon, 1996; Taylor, 2001). AChE is responsible for hydrolyzing the neurotransmitter acetylcholine; inhibition of the esterase causes accumulation of acetylcholine in synapses and excessive stimulation of muscarinic and nicotinic receptors (Figure 9.5B). Increased levels of acetylcholine overstimulate nicotinic and muscarinic receptors in the CNS, PNS, and neuromuscular junctions, causing a wide range of signs of poisoning. These signs may include changes in body temperature, heart rate, blood pressure, muscle twitching, or tremors. Death, although rare, is usually due to cessation of respiration due to anticholinesterase effects in both the CNS and PNS. Although organophosphates and carbamates both target acetylcholinesterase, nicotine acts as a direct cholinergic agonist rather than by cholinesterase inhibition; nicotine directly binds nicotinic receptors to hyperstimulate cholinergic neurons.

Both organophosphosphorus and carbamate compounds inhibit AChE activity by acting as pseudosubstrates. Unlike the natural substrate, these compounds remain in the active site of the enzyme for much longer periods of time, thereby preventing the enzyme from hydrolyzing its natural substrate, acetylcholine (Aldridge and Reiner 1969). Inhibition of AChE activity by organophosphorus insecticides is primarily a function of the electrophilicity of the phosphorus atom, with increased electrophilicity (increased partial positive charge) associated with greater rates of bonding to the negatively charged oxygen in the serine hydroxyl group. Thus, for organophosphorus insecticides, the binding of the organophosphorus compound to AChE is reasonably fast. The hydrolysis of the organophosphorus esters, however, is very slow, leading to an accumulation of phosphorylated AChE. Phosphorylated AChE is, for all practical purposes, irreversibly inhibited due to the very low reversibility of the enzyme activity.

Recovery of active AChE activity is dependent on the synthesis of new enzyme. In some cases, phosphorylated enzymes undergo an aging reaction when an alkoxy or aryloxy R group is dealkylated or dearylated and results in a negatively charged monoalkyl or aryl enzyme. In such instances, the hydrolysis step is no longer possible; thus, the enzyme is irreversibly altered. Kinetic studies on the inhibition of AChE activity, including aging and recovery, have been reported in a variety of fish species (Carr et al., 1995; Johnson and Wallace, 1987; Straus and Chambers, 1995; Wallace and Herzberg, 1988). Typically, recovery of AChE activity in fish is slower than that observed in mammals.

Inhibition of AChE activity by carbamate insecticides is a function of the carbamylation of the enzyme. The kinetics of this inhibition are slightly different from that observed with organophosphorus insecticides. Hydrolysis of carbamates (i.e., decarbamylation), while significantly slower than that observed for acetylcholine, is much more rapid than that observed with most organophosphorus compounds; thus, although AChE inhibition elicited by an organophosphorus pesticide is, in effect, irreversible, carbamate inhibition of esterase activity may be reversed. Furthermore, aging of the carbamylated enzyme does not occur.

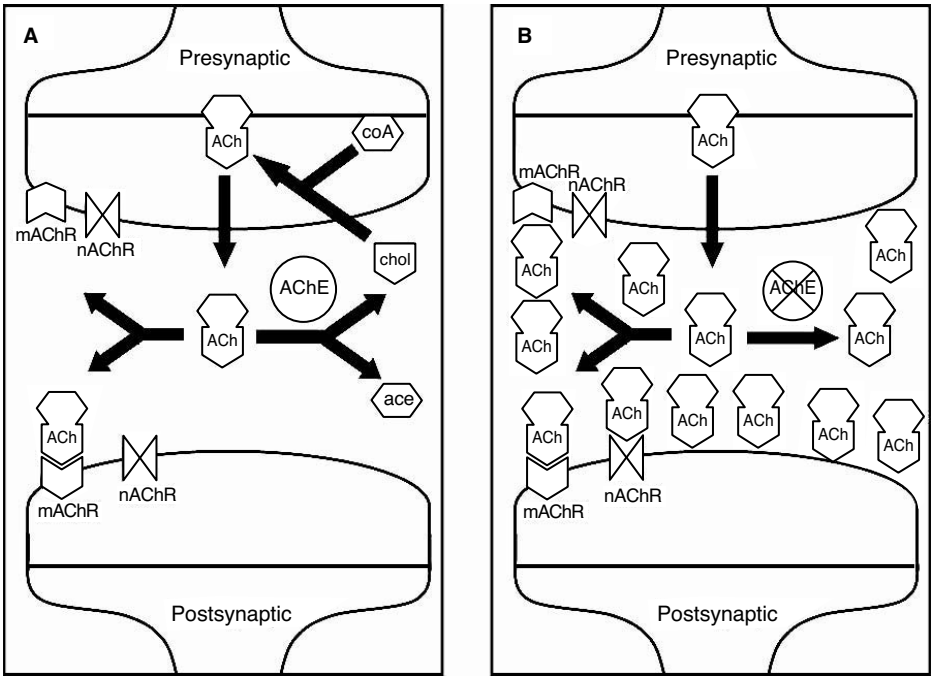

FIGURE 9.5 Role of acetylcholinesterase at synaptic junctions. (A) Typical cholinergic synapse. Acetylcholine is secreted by the presynaptic neuron into the synapse, where it binds to presynaptic and postsynaptic muscarinic and nicotinic receptors. Acetylcholinesterase breaks down acetylcholine into cholinesterase and acetylcholine. Cholinesterase is transported to the presynaptic neuron, where acetyl coenzyme A converts it into acetylcholine. (B) Effect of acetylcholinesterase inhibitors on cholinergic synapses. Acetylcholine is secreted into the synapse but is not broken down by acetylcholinesterase, leading to an excess amount of acetylcholine in the synapse. ACh, acetylcholine; AChE, acetylcholinesterase; mAChR, muscarinic receptor; nAChR, nicotinic receptor; chol, cholinesterase; coA, acetyl coenzyme A; ace, acetate.

Interestingly, although anticholinesterase compounds all disrupt acetylcholine signaling, all anticholinesterase compounds do not produce identical responses to poisoning (reviewed in Pope, 1999). Acetylcholine signaling disruption is the first step in the acute neurotoxic response but is not sufficient to explain all manifestations of toxicity. Several secondary, non-acetylcholinesterase targets have been recognized (Casida and Quisad, 2004; Pope, 1999) and are thought to play instrumental roles in the ultimate effects of cholinergic neurotoxicants. Furthermore, disruption of acetylcholine signaling alone may not fully explain the developmental neurotoxicity of these compounds. Careful consideration should be given to the non-cholinergic targets of these compounds when assessing toxicity, particularly in the developing embryo. As shown in Figure 9.5A, the biochemical mechanisms of acetylcholine production, secretion, and transmission are known in great detail. For the purposes of this discussion, only acetylcholine pathway components targeted by cholinergic agonists are addressed.

Manifestations of Cholinesterase Inhibitor Neurotoxicity in Fish

Brief exposures to organophosphorus pesticides can produce long-lasting neurological effects because of the irreversibility of AChE inhibition; in fact, depression of AChE activity is considered a primary indicator of organophosphate pesticide exposure in fish. As a result, the extensive research in fish neurotoxicity has sought to correlate acute toxicity in adult fish with level of acetylcholinesterase inhibition (Heath, 1995; Murty and Ramani, 1992; Zinkl et al., 1991). Based on work in several different fish species and with several cholinergic poisons, it is generally accepted that 70 to 80% AChE inhibition is lethal.

Acute exposures to organophosphorus and carbamate insecticides, in a variety of species including goldfish (*Carassius auratus*), fathead minnows (*Pimephales promelas*), medaka (*Oryzias latipes*), and rainbow trout (*Oncorhynchus mykiss*), increased spontaneous locomotor activity with high incidences of

convulsions, spasms, tetany, scoliosis, lordosis, and hemorrhage in the vertebral column, presumably due to damage resulting from spasms (Bradbury et al., 1991a,b; Drummond and Russom, 1990; McKim et al., 1987; Rice et al., 1997; Saglio et al., 1996). Organophosphorus and carbamate insecticides have variable effects on cough rate. Chlorpyrifos (Bradbury et al., 1991a) and fenitrothion (Klaverkamp and Hobden, 1980) have been reported to increase cough response in rainbow trout, while malathion, carbaryl (McKim et al., 1987), and acephate (Klaverkamp and Hobden, 1980) did not. Increased cough rate, however, is not thought to be associated with AChE inhibition (Klaverkamp and Hobden, 1980). Spinally transected rainbow trout exposed to chlorpyrifos exhibited increased defecation and bile loss from the anal opening, consistent with muscarinic effects of AChE inhibition (Bradbury et al., 1991a).

In spinally transected rainbow trout (*Oncorhynchus mykiss*) exposed to carbaryl, malathion (McKim et al., 1987), or chlorpyrifos (Bradbury et al., 1991a), decreased heart rate, decreased gill oxygen uptake efficiency, and increased ventilation volume were observed. Decreased heart rate has been attributed to inhibition of the heart by the vagus nerve (cranial nerve X) through cholinergic synapses. Decreased oxygen uptake by the gills and a compensatory increase in ventilation volume have been proposed to be caused by continuous stimulation of neuromuscular junctions associated with sphincters at the base of the efferent filamental arteries to secondary lamellae of the gill. The resulting vasoconstriction is thought to reduce blood flow to the lamellae, effectively reducing respiratory surface area and oxygen uptake efficiency (McKim et al., 1987; Pavlov, 1994).

Despite the wealth of acute toxicity data, relatively little is known about the developmental neurotoxicity of cholinergic agonists in fish. Existing data demonstrate that exposure either *in ovo* or as juvenile fish has detrimental consequences on learning and motorneuron development. Chlorpyrifos exposure produced hypoactivity in zebrafish (*Danio rerio*) hatchling swimming behavior (Levin et al., 2004). In addition, developmental chlorpyrifos exposure of zebrafish embryos has long-term effects on learning. Adult zebrafish exposed to chlorpyrifos during development show reduced choice accuracy and spatial discrimination (Levin et al., 2003). Behavioral effects have been seen in juveniles of other fish species as well. At concentrations of carbaryl or chlorpyrifos up to 10 times lower than 48-hour LC_{50} values, larval medaka (*Oryzias latipes*) were more susceptible to predation, although a consistent dose–response relationship between carbaryl exposure and susceptibility to predation was not observed (Carlson et al., 1998). *In vivo* electrophysiological studies of sublethal chlorpyrifos and carbaryl effects on the Mauthner cell startle response in larval medaka (Carlson et al., 1998) demonstrated an effect on neuromuscular junctions, as evidenced by a dose-related increase in the ratio of startle response to stimuli. An increase in motorneuron to muscle delay with increased exposure concentration was also noted. Both responses are consistent with AChE inhibition in the neuromuscular junction.

Interestingly, evidence from zebrafish (*Danio rerio*) suggests that AChE inhibition may not be the sole mechanism of developmental neurotoxicity. Developmental exposure to nicotine causes morphological changes in zebrafish hatchlings and impairs the swimming behavior and escape response (Svoboda et al., 2002). Using an *Islet1*-GFP transgenic zebrafish strain, nicotine was shown to delay development of spinal neurons and cause disruptions in axonal pathfinding of secondary motorneurons by nicotinic receptor activation (Svoboda et al., 2002). The molecular mechanism of developmental neurotoxicity remains unidentified for the majority of cholinergic agonists.

Pyrethroid Insecticides

Directed synthesis has produced insecticides derived from the natural pyrethrin esters of pyrethrum flowers (e.g., *Chrysanthemum cinerariifolium*) (Shafer et al., 2005). These synthetic, pyrethroid insecticides have greater stability in light and air than the natural pyrethrin esters yet maintain critical stereochemical characteristics required for alignment with target receptors (Soderlund et al., 2002). The synthetic pyrethroids are divided into two classes based on the presence or absence of a cyano group on the alpha carbon of the 3-phenoxybenzyl alcohol moiety (Figure 9.6). Type II pyrethroids all contain an α-cyano side group, while type I pyrethroids do not. Because of their potent insecticidal activity, low mammalian and avian toxicity, and varying levels of environmental stability (Bradbury and Coats, 1989; Clark, 1995), synthetic pyrethroids represent nearly 23% of the U.S. dollar value of the world insecticide market (Soderlund et al., 2002).

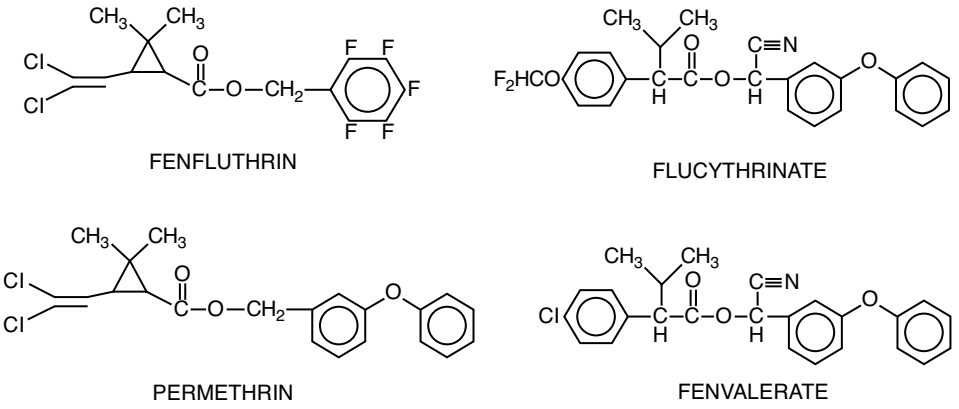

FIGURE 9.6 Examples of type I (fenfluthrin and permethrin) and type II (fenvalerate and flucythrinate) pyrethroid insecticides.

Mechanisms of Pyrethroid Neurotoxicity

In both mammals and insects, the primary mechanism of acute synthetic pyrethroid neurotoxicity is disruption of voltage-sensitive sodium channels (VSSCs) (Clark, 1995; Narahashi, 1992; Shafer et al., 2005; Soderlund et al., 2002). Mammalian VSSCs consist of a single α subunit and two β subunits (Shafer et al., 2005). The α subunit forms the channel pore, and the β subunits modify channel properties and membrane location (Figure 9.7A). Pyrethroid insecticides bind the α subunit and disrupt sodium regulation (Figure 9.7B,C). Type I pyrethroid insecticides prolong VSSC opening, allowing more sodium to cross the membrane and leading to repetitive firing of action potentials. Conversely, type II pyrethroids delay VSSC inactivation, resulting in a depolarization-dependent block that prevents action potential generation (Shafer et al., 2005; Soderlund et al., 2002). Although the primary mechanism of acute pyrethroid neurotoxicity is disruption of VSSCs, evidence suggests that numerous secondary sites and mechanisms of action are possible (Soderlund et al., 2002); for example, deltamethrin and resmethrin are toxic to *Paramecium tetraurelia*, although this organism does not have VSSCs (Soderlund et al., 2002). In this case, the synthetic pyrethoids were acting through calcium channels in the cilia membrane of the *P. tetraurelia* (Soderlund et al., 2002). Synthetic pyrethroids have also been shown to affect voltage-gated potassium and chloride channels, as well as ligand-operated channels such as the GABA receptor–ionophore complex, the nicotinic acetylcholine receptor, and the peripheral-type benzodiazepine receptor (Shafer et al., 2005; Soderlund et al., 2002). These effects, however, are usually associated with physiologically unrealistic pyrethroid exposures or nonspecific interactions. Both type I and type II pyrethroids cause repetitive firing in synapses, neuromuscular junctions, and the central nervous system. As a consequence, pyrethroid intoxication has been associated with releases of acetylcholine, GABA, dopamine, and norepinephrine. The type II compounds tend to elicit greater neurotransmitter releases than type I compounds because of enhanced ability to depolarize sensory and presynaptic nerve endings (Clark, 1995). In addition, perturbation of intraterminal calcium homeostasis, ATP-activated calcium sequestration, and responses of protein phosphorylation associated with calcium-dependent neurotransmitter release have also been suggested as playing a role in pyrethroid mode of action. Some of these effects may further accentuate neurotransmitter release caused by repetitive firing (Clark, 1995).

Unfortunately, there is little consensus on which, if any, of the acute neurotoxicity mechanisms are applicable to pyrethroid developmental neurotoxicity (Shafer and Meyer, 2004). Some pyrethroids insecticides show significant age differences in acute toxicity, with younger animals usually being more sensitive. Several studies also demonstrate persistent changes in motor activity, learning, and sexual activity following developmental pyrethroid exposure. None of these effects has been associated with a putative neurotoxic mechanism.

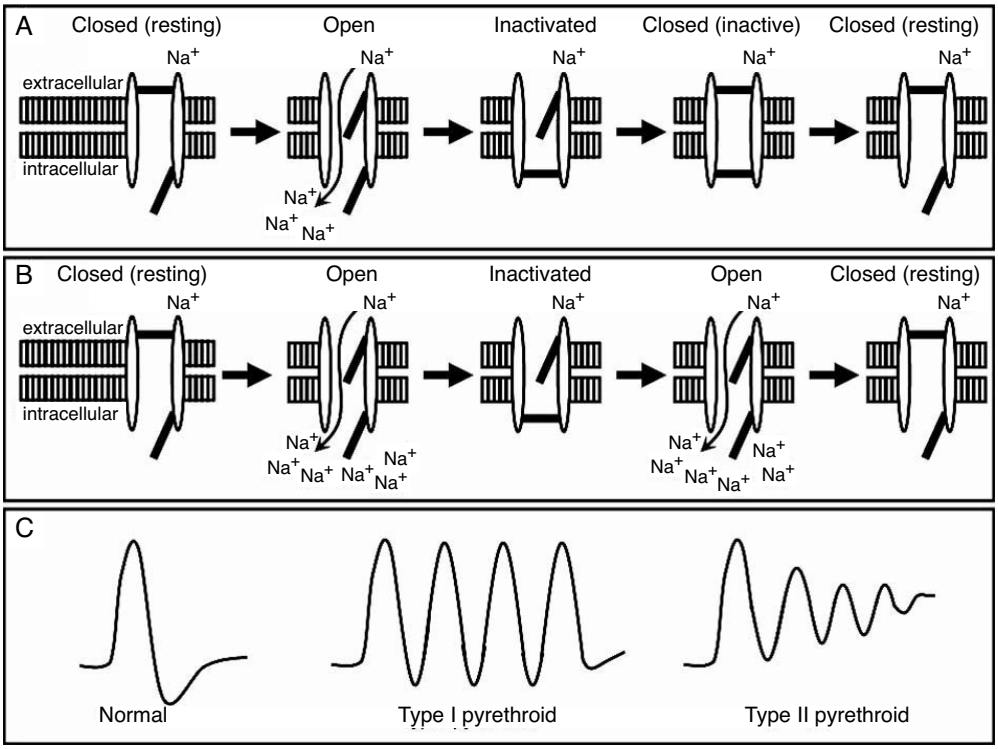

FIGURE 9.7 Effects of pyrethroids on voltage-sensitive sodium channels. (A) Firing cycle of a voltage-sensitive sodium channel (VSSC). At resting potential, the channel is closed to the extracellular environment. In response to a stimulus, the channel opens to allow sodium ions (Na^+) to flow into the cell. When the stimulus has passed, the channel closes the intracellular and extracellular gates to prevent Na^+ influx, ultimately returning to the resting state. (B) Pyrethroids can affect the voltage sensitive sodium channels in two ways. Type I pyrethroids prolong the opening of the sodium channel, leading to repetitive firing of action potentials. Type II pyrethroids prevent closing of the channel to an inactive state. (C) Comparing VSSC action potentials of normal, type I pyrethroid exposures, and type II pyrethroid exposures. Whereas type I pyrethroids lead to repetitive action potentials firings, type II pyrethroids show a gradual reduction in action potential amplitude, although never returning to the resting state.

Manifestations of Pyrethroid Neurotoxicity in Fish

Although synthetic pyrethroids have minimal mammalian and avian toxicity, they are very toxic to fish (Bradbury and Coats, 1989). Because of their high lipophilicity, the synthetic pyrethroids are readily absorbed through the gills (Baser et al., 2003; Polat et al., 2002). Fish lack at least one enzyme that metabolizes pyrethroids, meaning that metabolic turnover is particularly slow (Baser et al., 2003; Tilak et al., 2003). As a result, fish are particularly susceptible to pyrethroids entering the aquatic ecosystem. An extensive amount of research has focused on determining acute toxic dosage in adult fish (Baser et al., 2003; Das and Mukherjee, 2003; David et al., 2004; Polat et al., 2002; Rebach, 1999; Saha and Kaviraj, 2003; Tandon et al., 2005; Tilak et al., 2003; Tripathi and Verma, 2004).

As reviewed by Bradbury and Coats (1989), acute pyrethroid intoxication in small aquarium fish typically causes loss of schooling behavior, followed by hyperactivity, erratic swimming, violent whole-body seizures, and loss of buoyancy. Consistent with the steep dose–response relationships typically observed for pyrethroids, Carlson and coworkers (1998) failed to note significant effects on medaka (*Oryzias latipes*) susceptibility to predation or electrophysiological responses associated with the Mauthner-cell-mediated startle response at sublethal levels. Spinally transected rainbow trout (*Oncorhynchus mykiss*) exhibited an elevated cough rate shortly after exposure (Bradbury et al., 1991a). Increased cough rates were typically associated with an elevated secretion of mucus. Further intoxication resulted in increased hyperexcitability followed by tremors that progressed to seizures anterior to the site of the

transection. During seizures, the opercula were flared and in a state of tetany. Prior to death, seizures subsided and fish became inactive. Similar responses have been noted in bluegill (*Lepomis macrochirus*) (Bradbury et al., 1987; Little et al., 1993). The stages of behavioral changes in fish are generally consistent with those observed in mammals (Bradbury and Coats, 1989); however, an insufficient number of compounds have been studied in fish to differentiate pyrethroid intoxication syndromes as has been done with mammals (Shafer et al., 2005; Soderlund et al., 2002). Because seizures are typically stimulus dependent in hypersensitized fish, it seems reasonable to assume that pyrethroid-induced coughs could trigger convulsions. The cough response itself could be a CNS-mediated component in the seizure syndrome, a side effect due to interactions with sensory receptors in the pharynx and gill arches, or direct irritation of gill tissue. Both fenvalerate and permethrin have been shown to cause gill damage consistent with irritation (Bradbury et al., 1987; Kumaraguru et al., 1982). As reviewed by Clark (1995), pyrethroids have also been reported to cause transient dermal tingling, itching, and burning in humans and irritation to the mucous lining of respiratory passages.

Several measures of metabolic activity have been measured in fish exposed to pyrethroids. In general, protein levels and various dehydrogenases were reduced in response to pyrethroid exposure, while erythrocyte production was increased (Das and Mukherjee, 2003; Kumar et al., 1999; Tripathi and Verma, 2004). These data were interpreted as consequences of failed pyrethroid metabolism. Additionally, marked respiratory–cardiovascular effects observed in pyrethroid-intoxicated rainbow trout (*Oncorhynchus mykiss*) are consistent with increased muscular activity associated with seizures (Bradbury et al., 1991a). Increased ventilation volume was associated with moderate declines in oxygen uptake efficiency and therefore nearly constant oxygen consumption. Arterial blood oxygen levels were initially elevated but then declined dramatically, as did carbon dioxide levels and pH. Overall, these responses suggest a shift to anaerobic metabolism. These shifts in respiratory–cardiovascular and blood-chemistry parameters have also been reported in mammals and are also generally attributed to increased muscular activity (Bradbury and Coats, 1989).

Unlike studies of acute toxicity, few studies have addressed the developmental neurotoxicity of synthetic pyrethroids in fish. Exposure to sublethal levels of permethrin *in ovo* caused a hatching delay in medaka (*Oryzias latipes*). Medaka hatchlings demonstrated hyperactivity, uncoordinated movement, and an inability to respond to stimuli. These hatchlings also failed to inflate their swimming bladder and had spinal curvatures (González-Doncel et al., 2003). Unfortunately, these effects have yet to be correlated with a putative neurotoxicity mechanism, so it is unclear how pyrethroids may be inducing developmental neurotoxicity.

Organochlorine Insecticides

The organochlorine insecticides are among the largest category of insecticides and are used worldwide for public health (e.g., mosquito control) and agricultural production (Figure 9.8). The organochlorine insecticides are comprised of four distinct structural classes, which tend to be associated with unique mechanisms of action: (1) chlorinated ethane derivatives, (2) cyclodienes, (3) polychlorobornanes, and (4) lindane. Use of organochlorines has declined dramatically because of insecticide resistance and environmental concerns. Due to their persistence in the environment and their continued use in some parts of the world, organochlorine residues in sediments, soils, and biota are still observed (Woolley, 1995).

Mechanisms of Organochlorine Insecticide Neurotoxicity

As with synthetic pyrethroid insecticides, the neurotoxicity mechanism of chlorinated ethanes is the disruption of VSSCs. Studies with invertebrate and vertebrate preparations established that dichlorodiphenyltrichloroethane (DDT) prolongs the falling phase of the action potential, which typically produces repetitive firing. This repetitive neuron firing leads to the hyperexcitability and tremor noted in intoxicated insects, mammals, birds, and fish. Studies by Narahashi (1994) demonstrated that DDT caused voltage-sensitive channels to remain in the open state longer than normal and close slowly resulting in an increased overall open time. As reviewed by Woolley (1995), it appears that the type I

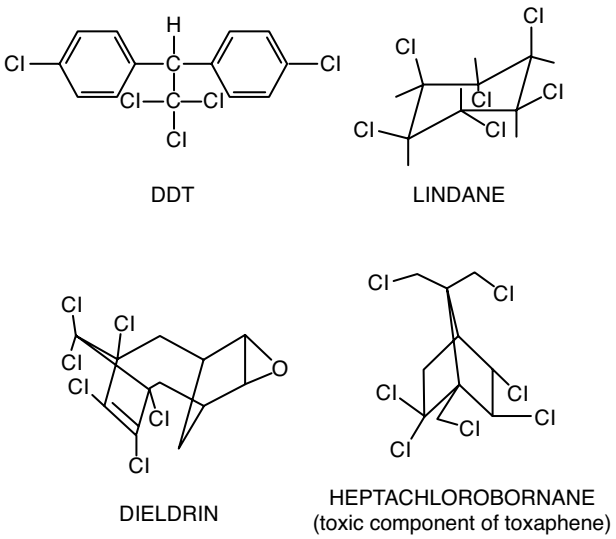

FIGURE 9.8 Examples of organochlorine insecticides.

pyrethroids and DDT likely affect the same target site on the sodium channel. Hyperexcitability is also associated with increased releases of neurotransmitters throughout the mammalian nervous system which likely reflect secondary responses but could represent an additional neurotoxic mechanism.

In mammals, the cyclodienes and polychlorobornanes cause anorexia, salivation, vomiting, and convulsions. During convulsions, insect and amphibian preparations exhibit excessive uptake of calcium into synaptosomes. This synaptosomal calcium uptake is associated with increased release of neurotransmitters. Subsequently, it was shown that cockroach strains resistant to cyclodiene insecticides, lindane, and toxaphene were also resistant to picrotoxin. Picrotoxin acts at one of the receptor sites in the GABA receptor–ionophore complex and is thought to block the channel. Normally, the GABA-gated chloride channel opens upon GABA binding, causing an increased flow of chloride, neuronal hypopolarization, and depression of excitability. Picrotoxin blockage of the GABA receptor eliminates normal chloride modulation of neuronal activity, causing hyperexcitability and, potentially, even convulsions. As these toxic effects are also induced by cyclodienes and polycholorbornanes, it was hypothesized that organochlorines may act by blocking GABA receptors. Confirmatory studies with insect and mammalian preparations established that several cyclodiene insecticides, toxaphene, and lindane act specifically at the picrotoxin site of the GABA receptor. In addition, binding at the picrotoxin site of the GABA receptor tends to correlate with toxic potency. Furthermore, these compounds inhibit GABA-stimulated flows of chloride across membrane vesicles (see reviews by Coats, 1990; Woolley, 1995).

Lindane is the gamma isomer of hexachlorohexane; it is approximately 10 times more acutely toxic to the rat than the alpha isomer and roughly 100 times more potent than the beta and delta isomers. The gamma isomer is strongly excitatory in both insect and mammalian nervous systems, while the alpha, beta, and delta isomers are weakly active or have depressant effects. At acute doses, lindane increases neuronal activity and neurotransmitter release. In addition to convulsant effects, lindane also causes anorexia, diarrhea, and hyperthermia in the rat. As discussed above, lindane is thought to antagonize GABA receptors in the CNS and the gastrointestinal tract. Consistent with this hypothesis, toxic effects of lindane can be attenuated or eliminated by pretreatment with GABA receptor agonists (Woolley, 1995).

Although disruption of VSSCs and GABA receptors are well characterized mechanisms of acute organochlorine neurotoxicity, there is little evidence for the role of these mechanisms in developmental neurotoxicity. Lindane is known to inhibit mammalian embryonic development from the eight-cell stage up to the blastocyst stage (Scascitelli and Pacchierotti, 2003). Several potential mechanisms of developmental neurotoxicity have been proposed, including disruption of endocrine homeostasis and alterations in intercellular communication (Scascitelli and Pacchierotti, 2003).

Manifestations of Organochlorine Insecticide Neurotoxicity in Fish

Organochlorine exposure affects behavior in many fish species. In unrestrained fish, cyclodiene exposure caused hyperactivity in response to stimuli, followed by recurrent tremors, rapid pectoral fin movement, and convulsions (Carlson et al., 1998). In spinally transected rainbow trout (*Oncorhynchus mykiss*), endosulfan and endrin intoxication induced branchial tremors, increased cough rate, and increased pectoral fin movement, with eventual tetany and convulsions anterior to the site of the transection (Bradbury et al., 1991a). Hyperactivity and increased cough frequency have also been reported with DDT intoxication in a number of fish species (Heath, 1995; Murty, 1986). DDT-induced hyperactivity is thought to contribute to decreased schooling behavior (Murty, 1986). At sublethal concentrations of endosulfan, medaka (*Oryzias latipes*) are less susceptible to predation, presumably due to hyperactivity; however, at endosulfan concentrations approximating the LC_{20} level, medaka are more susceptible to predation than control fish (Carlson et al., 1998). Respiratory–cardiovascular responses of cyclodiene-intoxicated rainbow trout included increased cough rate and ventilation volume, with no change in oxygen uptake efficiency, resulting in an increase in oxygen consumption. In a consistent manner, arterial blood oxygen remained near control levels until near death, while arterial blood carbon dioxide and pH levels decreased only slightly. Overall, the seizure activity in the cyclodiene-exposed trout was not associated with a shift to anaerobic metabolism. These responses were similar to those elicited by uncouplers of oxidative phosphorylation and may suggest a secondary effect associated with inhibition of ATPase activity (Bradbury et al., 1991a). Increased oxygen consumption has also been reported in a number of species following exposure to DDT and methoxychlor (Murty, 1986). *In vivo* electrophysiological studies of sublethal endosulfan exposures in medaka demonstrated increased motorneuron amplitude peaks and significantly increased stimulus-response ratio on the Mauthner cell startle response. The hyper-responsiveness of the Mauthner cell to stimuli is consistent with cyclodiene acting at the picrotoxin site in the GABA receptor–chloride complex. Furthermore, GABA is an important afferent inhibitory neurotransmitter to the Mauthner cell (Carlson et al., 1998).

Ethanol

Ethanol is a well-known neurotoxicant in mammals. Women who consume large amounts of ethanol during pregnancy often give birth to children exhibiting phenotypic abnormalities, collectively referred to as the fetal alcohol syndrome (FAS). These anomalies include growth deficiency, cognitive impairment, and distinctive craniofacial features (Coles and Platzman, 1993). The developmental potency of alcohol consumption has led to intensive investigation into the mechanisms of ethanol neurotoxicity, with particular emphasis on developmental neurotoxicity.

Mechanisms of Ethanol Neurotoxicity

Although many mechanisms have been postulated for the toxic effects of ethanol to the adult nervous system, ethanol is thought to produce neurotoxic effects mainly through interaction with the glutaminergic system, binding to the NMDA receptor and possibly interfering with the normal interaction of glycine with that receptor (Tsai and Coyle, 1998). Ethanol is a well-known developmental neurotoxicant in humans and laboratory animals. The mechanism for the toxic actions of ethanol on the developing nervous system is, however, unknown (Goodlett et al., 2005). Many hypotheses exist, including interaction with neurotrophins (Kentroti, 1997), cell-adhesion molecules (Bearer, 2001), or specific receptors (Costa and Guizzetti, 2002); increased apoptosis (Olney et al., 2002a,b); or increased oxidative stress (Cohen-Kerem and Koren, 2003). Very few studies have delved into the mechanisms of the effects of ethanol on the developing fish; however, it has been suggested that prechordal plate migration may be preturbed in ethanol-treated zebrafish (*Danio rerio*) embryos (Blader and Strähle, 2000).

Manifestations of Ethanol Neurotoxicity in Fish

Ethanol affects the function of the adult fish nervous system (Dlugos and Rabin, 2003; Gerlai et al., 2000), producing hyperactivity or hypoactivity (depending on dose), aggression, and changes in the

startle response. Studies of ethanol-induced neurotoxicity in zebrafish (*Danio rerio*) have shown that ethanol is a teratogen that produces developmental delay (Reimers et al., 2004), craniofacial abnormalities (Bilotta et al., 2004; Blader and Strähle, 2000; Carvan et al., 2004), changes in eye development (Bilotta et al., 2002, 2004), cell death (Carvan et al., 2004; Loucks and Carvan, 2004), and behavioral changes (Carvan et al., 2004). Interestingly, the occurrence or severity of these effects varies with the particular strain of zebrafish exposed (Loucks and Carvan, 2004).

Metals

The neurotoxicological effects of metal exposures must be considered in context of the chemical form of the metal, route and duration of exposure, and toxicokinetics. Effects observed in the field also require consideration of sediment and water quality characteristics associated with bioavailability (Dopp et al., 2004; Rüdel, 2003). Sources of neurotoxic metals in aquatic ecosystems include atmospheric deposition, point-source discharges, and non-point-source releases. Cadmium is primarily released through petroleum refining, fossil fuel combustion, and the use of cadmium in copper and nickel smelting (Wren et al., 1995). Mercury inputs are typically associated with atmospheric inputs from the combustion of fossil fuels and acid rain, point-source discharges from industry and gold-mining operations, and non-point-source discharges from agricultural use of organomercury seed treatments. Organotin and copper compounds have been input directly into aquatic systems as biocides in the shipping, fishing, and aquaculture industry (Blunden and Chapman, 1986; Rüdel, 2003). Globally, lead inputs include wet and dry atmospheric deposition, sewage treatment effluents, industrial and mining discharges, and non-point releases from mine tailings and highways (Gidlow, 2004; Pain, 1995). Aluminum loading to aquatic ecosystems is primarily associated with acidification and resulting releases from rocks, soils, and sediments (Lukiw and McLachlan, 1995).

Mechanisms of Metal Neurotoxicity

Generally, organismal-level effects elicited by metals are derived from multiple mechanisms. In addition to direct neurotoxic mechanisms, metals can damage respiratory surfaces and interfere with energy metabolism, osmoregulation, and endocrine function (Heath, 1995; Weber and Spieler, 1994). The extent to which metals are acting through neurotoxic mechanisms of action is a function of the metal species, dose, and route of exposure. Comprehensive reviews of primary scientific literature on metal toxicology (Chang, 1996) and neurotoxicology (Chang and Dyer, 1995) have been published, based primarily on mammalian model organisms. Below are brief overviews of neurotoxicity mechanisms for some selected metals whose neurotoxic mechanisms have been well studied.

A long environmental half-life and wide range of organ toxicity make cadmium one of the most toxic metals in the environment (Patrick, 2003). Several mechanisms have been suggested for the acute neurotoxicity of cadmium. Cadmium is thought to act as a calcium channel blocker or to bind calmodulin. As a result, cadmium disrupts neuromuscular junctions by blocking calcium-mediated neurotransmitter release (Beauvais et al., 2001). In the brain, cadmium inhibits magnesium and sodium/potassium ATPases, disrupting neurotransmitter uptake (Beauvais et al., 2001). In addition, cadmium effects on phospholipid metabolism in synaptic membranes, axonal transport, and basal adenylate cyclase activity have been reported. Cadmium-mediated disruption of cellular antioxidant defense mechanisms has also been proposed to increase neuronal damage through oxidative stress (Hastings, 1995).

Although all forms of mercury are neurotoxic to humans and animals, the most toxic forms are organic mercury compounds (Gochfeld, 2003). Organic mercury compounds are more lipophilic than elemental mercury or inorganic mercury compounds and therefore bioaccumulate in animal tissues (Shafer, 2000). Of the organic mercury compounds, methylmercury has been particularly well studied. Accumulation of methylmercury in the CNS and primary sensory tracts of mammals causes degeneration of visual cortices, dorsal root ganglia fibers, and the cerebellum. Numerous mechanisms have been proposed to explain the neurotoxicity of methylmercury in vertebrates, including disruption of the cell cycle and induction of apoptosis. Subcellularly, methylmercury is hypothesized to disrupt microtubule assembly and alter ion channel function. At the molecular level, cation homeostasis disruption and interference

with synaptic transmission have been hypothesized to mediate methylmercury neurotoxicity. Additionally, methylmercury is highly reactive with sulfhydryl groups (Shafer, 2000). Binding of methylmercury to sulfhydryl groups in critical proteins or macromolecules has been hypothesized to disrupt protein, DNA, and RNA metabolism; to perturb Ca^{2+} homeostasis; to induce oxidative damage; and to alter protein phosphorylation. Many of these mechanisms are interrelated and likely interact to produce methylmercury neurotoxicity (Chang and Verity, 1995).

Organic tin compounds are largely used as plastic stabilizers, although the major route of environmental exposure is from antifouling paints and pesticides (Rüdel, 2003). Organic tin compounds are hydrophobic and significantly more toxic than either elemental tin or inorganic tin compounds (Rüdel, 2003). Neurotoxic effects of organic tin compounds are thought to occur by a variety of interacting mechanisms; for example, triethyltin is hypothesized to uncouple oxidative phosphorylation, inhibit ATPase and phosphodiesterase activity, and initiate oxidative stress via ethane or ethylene metabolites, resulting in myleinic edema. Methyltin has been proposed to suppress mitochondrial respiration by inhibition of oxidative phosphorylation. The resulting hypoxic condition is thought to produce intracellular edema, glutamate release, and subsequent neuronal excitation. This excitation triggers neuron hyperstimulation and subsequent neuronal injury and death in the rat hippocampus (Chang and Dyer, 1995; Dopp et al., 2004).

Lead is a potent neurotoxicant in humans and animals, associated with impaired perception of spatial organization and increased distractibility (Bressler et al., 1999). A variety of mechanisms have been proposed for lead neurotoxicity, including vascular and neuroglia injury, oxidative stress, alterations in transcription, blockage of neurotransmitter release, and failure of neuronal migration (De Gennaro, 2002). A common component of these neurotoxic mechanisms is the ability of lead to mimic biologically relevant metals and cations, particularly calcium (Bressler et al., 1999; Costa et al., 2004; Marchetti, 2003). Lead mimicry of calcium has two primary consequences leading to neurotoxicity (Marchetti, 2003). Initially, lead competes with calcium for binding sites on calcium-regulated proteins such as calmodulin, synaptotagmin, and cadherin, disrupting gene expression, proteomic synaptic machinery, and neuronal migration (Bressler et al., 1999; De Gennaro, 2002; Marchetti, 2003; Prozialeck et al., 2002). Binding-site competition displaces calcium ions, subsequently exposing cells to higher levels of free calcium and potentially leading to oxidative stress (De Gennaro, 2002) and impaired myelination (Chang, 1996). The second method by which lead mimicry of calcium induces neurotoxicity is the disruption of calcium transport. Disruption of chloride ion exchange causes hyperexcitation of neurons (Chang, 1996). Lead also blocks calcium ion flux across dopamine receptors, glutamate receptors, and voltage-sensitive calcium channels (VSCCs). This blockage affects neurotransmitter synthesis and release, ultimately leading to neuron hyperexcitability and damage (Chang, 1996; Chang and Verity, 1995; Cory-Slechta and Pounds, 1995; De Gennaro, 2002; Marchetti, 2003).

Aluminum is a potent neurotoxicant in both animal brain and cultured neurons (Savory et al., 2003). Acute aluminum neurotoxicity disrupts blood–glia–neuron membranes and alters neuronal structure and function (Lukiw and McLachlan, 1995). Aluminum is of particular interest because some of its neurotoxic effects resemble Alzheimer's disease (Yokel, 2000). Similar to this late-onset neurodegeneration disease, acute aluminum neurotoxicity causes the formation and accumulation of insoluable β-amyloid, aggregation of hyperphosphorylated tau protein, and disruption of cortical cholingeric neurotransmission (Yokel, 2000). These effects arise from disruption of calcium binding (Yokel, 2000). Aluminum perturbance of calcium binding induces excessive calcium-ion influx in cholinergic neurons and mitochondria. This increase in mitochondria calcium influx causes apoptosis and may explain the significant loss of cholingergic neurons in Alzheimer's disease (Savory et al., 2003; Szutowicz, 2001). Significant pathological differences exist between Alzheimer's disease and aluminum neurotoxicity, making direct comparisons controversial (Yokel, 2000). Furthermore, several other molecular mechanisms have been proposed for aluminum neurotoxicity, including increased iron-induced oxidative stress, inhibition of Mg^{2+}-requiring enzymes, and modification of second-messenger systems (Lukiw and McLachlan, 1995; Yokel 2000).

Metals represent some of the most potent developmental neurotoxicants, particularly mercury and lead. Fetal methylmercury exposure in humans causes Minamata disease, characterized by cerebral palsy, mental retardation, and seizures (Davidson et al., 2004). Autopsies of Minamata patients reveal cerebral

and cortical brain lesions with peripheral neuropathy (Gochfeld, 2003). Furthermore, studies with model organisms suggest that low-level exposure to methylmercury may not manifest effects until much later in life (Davidson et al., 2004). Ethylmercury is thought to produce the same developmental effects as methylmercury and may be associated with the spectrum of autism, learning, and speech disorders (Davidson et al., 2004). The developing nervous system is also vulnerable to lead exposure. Children exposed to lead have poor coordination, behavioral problems, and reading disabilities (De Gennaro, 2002). Several aspects of the developing nervous system are sensitive to metal neurotoxicity. Increased absorption through an immature blood–brain barrier means that neurotoxicity may be induced at a much lower exposure level. Furthermore, sublethal metal exposure levels may alter key processes such as synapse formation, synapse refinement, and neurotransmitter release, causing effects that are manifested later in life (Marchetti, 2003). Although currently under investigation, the extent to which the acute metal neurotoxic mechanisms described above are involved in developmental neurotoxicity is not well understood.

Manifestations of Metal Neurotoxicity in Fish

Numerous reviews have summarized the behavioral responses of fish to metal intoxication (Atchison et al., 1987; Heath, 1995; Weber and Spieler, 1994). In addition to direct neurotoxic mechanisms, alterations in avoidance or attraction responses, activity patterns, critical swimming speed, respiratory behavior, intraspecific social interactions, reproduction, feeding, and predator avoidance (Atchison et al., 1987) can be attributed to direct damage to respiratory surfaces and interference with energy metabolism, osmoregulation, and endocrine function (Heath, 1995; Weber and Spieler, 1994). Examples provided below attempt to link neurophysiological or behavioral responses to neuropathological or biochemical alterations. The extent to which the neurotoxic mechanisms discussed above are relevant in fish remains to be assessed (Weber and Spieler, 1994). Studies of chemoreception have quantified the extent to which metals attract or repel fish and the extent to which metals affect responses to endogenous chemical signals such as pheromones. Alterations in avoidance or attraction responses have been observed in response to a number of metals, including cadmium, copper, and mercury. In rainbow trout (*Oncorhynchus mykiss*), lake whitefish (*Coregonus clupeaformis*), Atlantic salmon (*Salmo salar*), and goldfish (*Carassius auratus*), copper induces avoidance behavior (Atchison et al., 1987). This avoidance behavior is attributed to the effects of copper on the olfactory bulb. Copper attenuates electrical responses of the olfactory bulb and receptor cells to excitatory compounds (Hara et al., 1976; Sutterlin and Sutterlin, 1970; Winberg et al., 1992). Furthermore, copper exposure causes degeneration of specific olfactory receptor cells (Brown et al., 1982; Julliard et al., 1993), likely through oxidative-stress-mediated apoptosis (Julliard et al., 1993, 1996). Interestingly, oxidative stress may be partly responsible for the observed neurological effects in Wilson's disease, a genetic defect in copper metabolism leading to copper neurotoxicity in humans (Bondy, 1996). In fish, cadmium exposure has been correlated to changes in brain acetylcholinesterase activity, although these neurochemical changes have not been correlated with changes in swimming behavior in larval rainbow trout (Beauvais et al., 2001).

Although many metals elicit an avoidance response, mercuric chloride and methylmercury attract fish (Atchison et al., 1987; Heath, 1995). Exposure of mercuric chloride and methylmercury to the olfactory bulb and receptors of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) depressed electrical responses (Baatrup et al., 1990; Hara et al., 1976; Sutterlin and Sutterlin, 1970). Methylmercury has also been found to preferentially accumulate in olfactory receptors and the olfactory nerve of Atlantic salmon following dietary exposure (Baatrup et al., 1990; Berntssen et al., 2003). Chronic dietary exposure reduced overall activity in the Atlantic salmon (*Salmo salar*) and caused preferential histopathological damage to the brain stem (Berntssen et al., 2003).

Exposure to tributyltin oxide causes a variety of locomotor effects in fish. Rainbow trout (*Oncorhynchus mykiss*) exposed to tributyltin oxide swam longer distances at higher velocities but with erratic swimming tracks, indicating a loss of orientation (Triebskorn et al., 1994). In addition, intoxicated trout had a depressed startle response and were unresponsive to external stimuli. Similar behavioral and locomotor responses have been noted in minnows (*Phoxinus phoxinus*) (Fent and Meier, 1992). Tributyltin

oxide induces cytopathological damage in the brain and eyes of several fish (Fent and Meier, 1992; Triebskorn et al., 1994; Wester and Canton, 1987). Pathological lesions observed in the optic tectum and eyes suggest that the optic system is a putative target of tributyltin (Fent and Meier, 1992; Triebskorn et al., 1994; Wester and Canton, 1987). In support of this hypothesis, ultrastructural alterations have been found in the optic tectum of tributyltin-oxide-exposed rainbow trout (Triebskorn et al., 1994).

Lead exposure caused a general increase in locomotor activity in mirror carp (*Cyprinus carpio*) (Rehman, 2003). In zebrafish (*Danio rerio*) and fathead minnows (*Pimephales promelas*), lead exposure reduced feeding ability, as evidenced by feeding miscues and increased prey-handling times (Nyman, 1981; Weber et al., 1991). This reduction in feeding ability was attributed to psychomotor coordination, based on correlations between increased brain lead levels, increased serotonin and norepinephrine concentrations, and decreased feeding ability (Weber et al., 1991). Lead exposure induced similar increases in brain serotonin and decreases in GABA in walking catfish (*Clarias batrachus*) (Katti and Sathyanesan, 1986). Yet, although lead increased brain serotonin and norepinephrine levels, lead exposure did not increase dopamine levels in fathead minnows (Weber et al., 1991).

The effects of aluminum on fish behavior have not been reported. Long-term exposure of rainbow trout (*Oncorhynchus mykiss*) to aluminum caused aluminum accumulation in the cerebrovascular endothelium and throughout the telencephalon (Exley, 1996). This finding suggests that aluminum is capable of crossing the blood–brain barrier in fish as well as in mammals (Lukiw and McLachlan, 1995; Szutowicz, 2001). Also, in the telencephalon of aluminum-exposed rainbow trout were dense extracellular deposits of aluminum apparently surrounded by a protein matrix, suggesting similar neuropathological responses in fish and mammals.

The effects of a number of metals on prey susceptibility, schooling behavior, and aggression have been studied across a wide array of species (Heath, 1995; Weber and Spieler, 1994). Although many of these adverse effects are hypothesized to reflect alterations in sensory receptors or the structure and function of the PNS and CNS, direct cause-and-effect relationships have not been established. Even less evidence is available for developmental neurotoxicity of metals in fish.

Neurotoxins

Through evolutionary pressure, plants and algae have developed a number of strategies to avoid consumption and maximize competitive advantages in accumulating resources. It has been estimated that approximately 7000 terrestrial plant species are toxic to animals, with perhaps one half of these species producing compounds targeting the nervous system. In some instances, plant neurotoxins have been exploited to facilitate fish harvesting (Bhatt, 1991). Several aquatic organisms produce fish neurotoxins and are of increasing concern in many areas (Burkholder et al., 1992). The following discussion provides an overview of the neurotoxicity of several specific neurotoxins and illustrates how these compounds are used to assess fish brain function.

Mechanisms of Neurotoxin Toxicity

Neurotoxins derived from aquatic organisms produce toxicity by a variety of mechanisms. Toxins derived from cyanobacteria, dinoflagellates, and coral act on membrane sodium channels directly or on membranes to create ion permeabilities. Such toxins are classified in terms of their ability to activate, stabilize, or occlude the sodium channel (see review by Strichartz and Castle, 1990). Toxins that activate sodium channels cause long-lasting sodium ion influx and correspondingly prolonged depolarization of excitable membranes, preventing complete inactivation. Failure of membrane repolarization also causes periods of rapid, spontaneous impulse firing (Strichartz and Castle, 1990). Brevetoxins and ciguatoxin, derived from the marine dinoflagellates *Ptychodiscus brevis* and *Gambierdiscus toxicus*, respectively, are among the most studied sodium-channel activator toxins. Toxins isolated from *P. brevis* bind VSSCs and cause activation at resting membrane potentials. Competitive binding studies have established that the most toxic brevetoxins bind preferentially at the active site within the sodium channel (Trainer et al., 1990). Frelin and coworkers (1990) have also substantiated that ciguatoxin acts as a sodium-channel activator and binds to the same active site as the brevetoxins.

Toxins stabilize sodium channels by inhibiting inactivation of the sodium current during depolarization, thus keeping the sodium channel in the open state. This sodium channel stabilization causes a prolonged period of calcium influx through VSCCs, causing increased calcium-mediated secretions and contractions (Strichartz and Castle, 1990). Toxins that stabilize sodium channels bind different sites than toxins that activate sodium channels; consequently, sodium-channel-stabilizer toxins can synergize with sodium-channel-activator toxins, causing larger membrane depolarizations at lower doses. Small peptides produced by anemones and larger proteins produced by mollusks in the family Conidae are examples of sodium-channel-stabilizer toxins. Sodium-channel-occluder toxins are small organic cations that are high-affinity, but reversible, blockers of the channel (Strichartz and Castle, 1990). The subsequent inhibition of sodium conductance renders excitable membranes inactive and halts impulse propagation. Tetrodotoxins and saxitoxins are classic examples of sodium-channel-occluding toxins. Tetrodotoxin is produced by some fish in the order Tetraodontiformes and the Costa Rican frog *Atelopus* (Ritchie and Greene, 1985). Saxitoxins are produced by marine dinoflagellates in the genera *Gonyaulax* and freshwater cyanobacteria in the genera *Anabaena* and *Aphanizomenon* (Carmichael, 1997). Some sodium-channel-occluding toxins discriminate between sodium-channel types; for example, the μ-conotoxins, from the mollusk *Conus geographus* potently block muscle sodium channels but only weakly inhibit sodium currents in neuronal and cardiac sodium channels.

Some toxins create ion permeabilities without affecting ion channels. Palytoxin, derived from coral, irreversibly increases cation permeability, perhaps by converting the sodium/potassium pump to a passive channel (Strichartz and Castle, 1990). Other aquatic neurotoxins have mechanisms that do not center on ion regulation; for example, cyanobacteria of the genera *Anabaena* and *Oscillatoria* produce anatoxins that disrupt acetylcholine function at neuromuscular junctions. Anatoxin-a acts as an acetylcholine mimic, binding nicotinic acetylcholine receptors at vertebrate muscle endplates, and is reported to be eight times more potent than acetylcholine. Furthermore, anatoxin-a is resistant to acetylcholinesterase hydrolysis, causing an overstimulation of muscle cells to the point of fatigue. The similarly named anatoxin-a(s) causes the same symptoms of neurotoxicity as anatoxin-a but by a different mechanism. Anatoxin-a(s) is a naturally occurring organophosphate that inhibits acetylcholinesterase activity in a manner similar to the organophosphate insecticides discussed previously.

In addition to aquatic toxins, terrestrial plant toxins can affect the CNS of vertebrates, including fish. Notable examples are the pyrethrins, derived from *Chrysanthemum cinerariifolium*, whose structures were subsequently modified synthetically to develop the pyrethroid insecticides, as summarized previously. Certain plant toxins have also been exploited by several cultures as an aid in harvesting; for example, piscicidal plants derived from plants of the Garhwall hills of India are of great ethnobiological importance. Bhatt (1991) has described how a flavonoid derived from *Engelhardtia colebrookiana* (Lindle) causes degeneration of neurons and neural tracts in the medulla oblongata of freshwater fish. Strychnine, an alkaloid derived from *Strychnos nux-vomica*, has long been known as a central nervous system stimulant in animals. Strychnine selectively antagonizes GABA in the brain and glycine in the spinal cord (Dorling et al., 1995) and has been exploited in fish neurotoxicology studies to elucidate the role of these inhibitory neurotransmitters.

Manifestations of Neurotoxin Toxicity in Fish

Brevetoxins and ciguatera toxins elicit similar effects in fish, consistent with their identical mechanisms of neurotoxicity summarized previously. Red tides caused by *Ptychodiscus brevis* brevotoxins are associated with massive fish kills. Exposure of ciguatera toxins to coney (*Epinephelus fulvus*), schoolmaster (*Lutjanus apodus*), mahogany snapper (*Lutjanus mahogoni*), largemouth bass (*Micropterus salmoides*), blueheads (*Thalassoma bifasciatum*) (Davin et al., 1986, 1988), and western mosquitofish (*Gambusia affinis*) (Lewis 1992) caused skin color variations, rapid opercular movement, inactivity, loss of equilibrium, erratic swimming, jerky feeding movements, loss of orientation, and death. Exposure to another toxic dinoflagellate, *Pfiesteria piscicida*, has been reported to induce sudden sporadic movement, disorientation, lethargy, and apparent suffocation followed by death in 11 species of fish, including striped bass (*Morone saxatilis*), southern flounder (*Paralichthys lethostigma*), Atlantic menhaden (*Brevoortia tyrannus*), and American eel (*Anguilla rostrata*) (Burkholder et al., 1992; Glasgow et al., 1995).

There are few reports of other aquatic neurotoxin effects in fish. Accumulations of saxitoxins, the toxins responsible for paralytic shellfish poisoning, and anatoxins in fish tissues have been documented primarily because they are monitored within the context of human health protection. Toxic endpoints of saxitoxins and anatoxins in fish, however, are rarely assessed. In mammals, including humans, tetrodotoxins and saxitoxins block vasomotor nerve function, ultimately causing death by paralysis of respiratory muscles (Ritchie and Greene, 1985). In laboratory studies, anatoxin-a and anatoxin-a(s) elicit acetylcholine and acetylcholinesterase inhibition responses in electric rays (*Torpedo californica*) and in electric eels (*Electrophorus electricus*) (Hyde and Carmichael, 1991; Mahmood and Carmichael, 1987; Swanson et al., 1991). Although anatoxin poisonings have been reported in livestock, domestic animals, and wild birds (Carmichael, 1997), effects on fish in the field remain largely undocumented.

Spinally transected rainbow trout (*Oncorhynchus mykiss*) exposed to strychnine (Bradbury et al., 1991b) rapidly exhibit increased cough rate as well as whole-body spasms that included tail arching. These spasms were similar to those elicited by polar narcotics. The strychnine-exposed rainbow trout, however, exhibited an elevated response to outside stimuli that was not observed with trout exposed to narcotics, acetylcholinesterase inhibitors, or cyclodiene and pyrethroid insecticides. Similar responses to strychnine exposure were also observed in sharks (Baldridge, 1969), medaka (*Oryzias latipes*) (Carlson et al., 1998; Rice et al., 1997), and fathead minnows (*Pimephales promelas*) (Drummond and Russom, 1990). Under acute exposure conditions to nonlethal concentrations of strychnine, medaka are less susceptible to bluegill (*Lepomis macrochirus*) predation, presumably due to heightened responses to outside stimuli (Carlson et al., 1998). Rainbow trout exposed to lethal strychnine concentrations had decreased ventilation frequency and ventilation volume, causing increased oxygen uptake efficiency and oxygen consumption (Bradbury et al., 1991b); consequently, arterial blood oxygen level decreased throughout these strychnine exposures and was associated with a drop in arterial blood pH and an increase in hematocrit. These respiratory–cardiovascular responses are consistent with anaerobic metabolism during strychnine-induced seizures. Decreased respiration and hypoxia have similarly been noted in mammals exposed to strychnine. In mammals, periods of impaired respiration during convulsions are thought to cause hypoxia, leading to medullary paralysis and death (Klaassen, 1996; Slater, 1965). Studies of the Mauthner cell startle response in strychnine-exposed medaka showed an increase in reflex response to stimuli. An associated delay between the Mauthner cell and motorneuron action potentials and minimal decline in the time between the motorneuron action potential and excitation of the tail musculature were noted (Carlson et al., 1998). These results in medaka are consistent with glycine antagonism and blockage of spinal cord motor cell inhibitory pathways, both of which are documented responses to strychnine exposure in the Mauthner cell soma and dendrites of several other fish species (Furukawa et al., 1964; Legendre and Korn, 1994).

Summary

The mechanisms and effects of neurotoxicants in fish have been studied with a wide variety of *in vitro* and *in vivo* methods. In addition to advancing understanding of fundamental neurobiology, this research helps develop techniques to assess adverse effects of neurotoxicants for ecological risk assessments. Of special note are those investigations that link structural and functional alterations of the nervous system at the subcellular or cellular level to physiological and behavioral effects. Research integrating information across levels of biological organization will further mechanistic understanding of neurotoxicants, allowing for extrapolation across species and chemicals and development of approaches to identifying ecologically relevant neurotoxic responses. The National Research Council (NRC, 1992) has observed that one of the greatest challenges in developing methods for neurotoxicology is associating neuromorphological, neurochemical, and neurophysiological alterations with behavioral changes. The *in vitro* and *in vivo* fish models described here are uniquely suited for characterizing chemical-induced neurotoxicity and understanding the biochemical, physiological, and morphological mechanisms underlying behavioral alterations.

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10

The Endocrine System

Peter Thomas

CONTENTS

Introduction

The endocrine, nervous, and immune systems are the primary means of communication between cells and organs in multicellular animals. The regulation of many physiological functions involves integration of nervous and endocrine systems, although typically endocrine responses are slower and longer lasting than neural ones. Specific chemical messengers, hormones, are secreted by endocrine glands directly into the blood. Although the hormones are distributed in the bloodstream throughout the body, they only act at specific target tissues. This specificity of hormone action is due to the presence of specific receptor molecules that preferentially bind the hormone in the target cells. Hormone binding activates the receptor, causing stimulation of intracellular second messenger pathways or direct binding of the hormone–receptor

complex to regulatory elements on genes, ultimately resulting in alterations in cell function. Some hormones, including the gonadal sex steroids, also function in a paracrine fashion by acting locally on surrounding target cells without entering the circulation.

Most of the information reported to date on endocrine toxicity indicates that xenobiotic chemicals primarily interfere with gonadal, thyroid, and adrenocortical (interrenal) functions in fish and other vertebrate species. Specific hormones secreted by the hypothalamus and pituitary regulate the activity of each of these endocrine glands; therefore, chemical interference with the endocrine axes controlling these three endocrine functions identified in laboratory studies are briefly reviewed in this chapter. Evidence for endocrine imbalances in fish populations exposed to endocrine-disrupting chemicals in the environment is covered in Chapters 22, 24, and 25. The current discussion is limited to evidence obtained in adult animals, although early developmental stages, such as the period of phenotypic sex determination, gonadal sex differentiation, and onset of puberty, appear to be especially sensitive to endocrine-disrupting chemicals (Gray and Metcalfe, 1997; Iguchi, 1999; Norrgren et al., 1999; Strussman and Nakamura, 2002). Most of this chapter focuses on the endocrine control of reproduction by the hypothalamic–pituitary–gonad axis and the sites and mechanisms of chemical disturbance of reproductive endocrine function, because the majority of the evidence for endocrine disruption in vertebrates has been obtained in this system. The literature on these topics is very extensive, so a comprehensive review of all the major contributions to these fields is not attempted here; thus, the emphasis of this review to a certain extent reflects the author's own research interests and findings.

Hypothalamic–Pituitary–Gonadal Axis

Hormones and other factors secreted by the hypothalamic–pituitary–gonadal (HPG) axis control the development of reproductive tissues and the initiation and precise coordination of the complex processes that occur during the annual reproductive cycle in teleost fish, culminating in the release and fertilization of mature gametes. The basic features of the HPG axis in fish are similar to those of other vertebrates and are shown in Figure 10.1. Environmental cues, such as changes in photoperiod, water temperature and chemistry, and social stimuli, as well as physiological changes such as alterations of nutritional state, are detected by peripheral and internal sensory systems, and the information is relayed via neural pathways to the hypothalamus and associated brain regions. The hypothalamus integrates this information, resulting in the secretion of various neurotransmitters and neuropeptides that influence the activities of gonadotropin-releasing hormone (GnRH)-producing neurons in the preoptic area and medial basal hypothalamus. GnRH is a decapeptide and the primary neurohormone that controls reproduction. The GnRH neurons directly innervate the gonadotropin-producing cells in the anterior pituitary (gonadotropes) of teleosts to regulate the synthesis and secretion of gonadotropins. The GnRH is released from nerve terminals in the vicinity of the gonadotropes and binds to specific receptors on the plasma membrane, resulting in activation of intracellular signaling pathways that regulate the release of gonadotropins. Monoamine neurotransmitters such as dopamine and serotonin also act on the pituitary to modulate the secretion of GnRH and gonadotropins (Figure 10.1). The neuroendocrine and intracellular second-messenger systems controlling gonadotropin secretion are briefly summarized in subsequent sections.

It is generally accepted that the seasonal reproductive cycle in teleosts, like that of tetrapods, is under dual gonadotropin control by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), previously named GTH I and GTH II, respectively, in teleosts. FSH and LH are glycoprotein hormones with molecular weights of approximately 28 to 30 KDa, and they are comprised of two subunits, an alpha subunit that is common to both gonadotropins and thyrotropin (thyroid-stimulating hormone) and a beta subunit that is hormone specific. The two gonadotropins are produced in different populations of gonadotropes within the anterior pituitary (adenohypophysis) and show different secretory patterns during the reproductive cycle in salmonids, the only teleost fishes in which FSH secretion has been investigated (Gomez et al., 1999; Swanson et al., 2003). LH and FSH bind to specific G-protein-coupled receptors on the surface of somatic cells in the gonads. In salmon, one of the gonadotropin receptors specifically interacts with homologous LH (GTH II) and is localized in granulosa cells in preovulatory ovarian

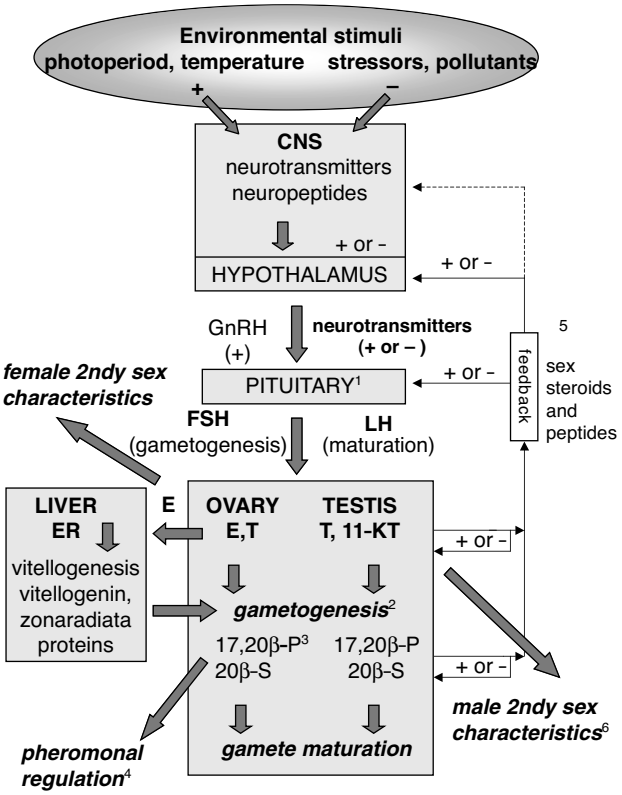

FIGURE 10.1 Schematic representation of the hypothalamic–pituitary–gonadal axis controlling reproduction in fish. [1]Growth hormone, prolactin, and thyroid hormones also influence teleost reproduction. [2]Only secondary (vitellogenic) oocyte growth is under hormonal control. [3]Either 17,20β-P or 20β-S is the primary progestin hormone in most species. [4]Conjugated metabolites of 17,20β-P and 20β-S in urine of periovulatory females influence reproductive behavior of conspecific males. [5]Feedback effects of steroids vary with the stage of the reproductive cycle; regulatory peptides include inhibin and activin. [6]Male secondary sex characteristics include male territorial behavior, gonopodia, breeding tubercles, and male coloration.

follicles and in Leydig cells in spermiating males (Miwa et al., 1994). The other receptor binds both LH and FSH (GTH I) and is present in both thecal and granulosa cells during vitellogenesis but is only present in the thecal cells in the preovulatory follicle. This receptor is present in the Sertoli cells in males at all stages of spermatogenesis (Miwa et al., 1994). The specificities of the gonadotropin receptors in other teleost species are currently unknown due to lack of purified homologous FSH. It was found that mammalian LH and FSH can activate all the receptors identified in African catfish and channel catfish to some extent (Kumar et al., 2001a,b; Vischer and Bogerd, 2003). Gonadotropin binding to the receptors results in activation of G-proteins, adenylyl cyclase, and calcium-dependent second-messenger signaling pathways (Kumar et al., 2001a,b; Miwa et al., 1994; Vischer and Bogerd, 2003). These intracellular signals subsequently cause alterations in the production and secretion of steroid hormones, growth factors, and regulatory peptides such as inhibin.

Steroid hormones are relatively stable structures, consisting of four fused hydrocarbon rings with oxygen and carbon substitutions at different positions that are characteristic for each class of steroid hormone. All steroid hormones are synthesized from a common precursor, cholesterol, via a series of biosynthetic steps catalyzed by different steroidogenic enzymes. The protein that regulates the transfer of cholesterol into the inner mitochondrial membrane, steroidogenic acute regulatory protein (StAR), is a key regulatory (rate-limiting) step in steroid synthesis whose production is upregulated by gonadotropins in the gonads and adrenocorticotropin in the adrenal cortex in mammals (Stocco and Clark, 1996) and in fishes (Kasukabe et al., 2002). Cholesterol is converted to pregnenolone, a steroid with 21 carbon

atoms (C-21 steroid) by the P450 enzyme cholesterol side-chain cleavage (P450scc). This enzyme is present on the inner mitochondrial membrane and has been characterized in teleosts (Takahashi et al., 1993). Pregnenolone, in turn, undergoes a series of isomerizations and hydroxylations by steroidogenic enzymes—3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase (P450c17), 21-hydroxylase (P450c21), 11β-hydroxylase (P450c11), and 20β-dehydroxysteroid dehydrogenase (20β-HSD)—to form the C-21 steroid hormones progestins and glucocorticoids (Nagahama, 2000). 17α-Hydroxyprogesterone is converted to androgens (C-19 steroids) by P450c17, which has 17,20 lyase activity (removes the side chain) and by 11β-hydroxysteroid dehydrogenase (11β-HSD) to produce 11-ketotestosterone. Androgens are subsequently converted to estrogens (C-18 steroids) by the aromatase enzyme (P450arom). The sex steroids (estrogens, androgens, and progestins) are produced in the theca and granulosa cells in the ovary and primarily in the Leydig or interstitial cells in the testis. Sex steroids have both endocrine and paracrine effects mediated by binding to specific receptors on distant target tissues, such as the liver and hypothalamus, and within the gonads themselves. Sex steroids are transported in the blood bound to sex-steroid-binding proteins (Laidley and Thomas, 1994, 1997). The pattern of steroidogenesis changes during the reproductive cycle in both males and females, from the production of estrogens and androgens during the period of gamete production (gametogenesis) to the production of progestins during gamete maturation and spawning.

Endocrine Control of the Reproductive Cycle

Reproductive activity in most fishes is seasonal, and the seasonality becomes more pronounced in fish that reproduce at higher latitudes. Both external environmental stimuli and endogenous circannual rhythms are thought to influence reproductive cycles in teleosts, although evidence of the latter has only been obtained for a few species. Photoperiod and temperature are the major environmental variables that influence reproductive cycles. Photoperiod changes initiate the reproductive cycle in most temperate species, whereas temperature changes often control the completion of the cycle to ensure that larval production coincides with favorable environmental conditions for their survival (Khan and Thomas, 1999). These environmental variables and other factors such as social cues influence the activity of the reproductive neuroendocrine system by neurochemical signals and neural pathways that have not been clearly delineated. In addition, the physiological functions of the two gonadotropins FSH and LH have not been as distinguishable in some fish species as they have been in mammals, based on the limited data available at present on teleost FSH physiology. FSH (GTH I) clearly has important roles in salmonids and several other fishes during early gonadal development and vitellogenesis or spermatogenesis, whereas LH (GTH II) regulates the final stages of the reproductive cycle including oocyte maturation, ovulation, and spermiation; however, a distinct physiological role for FSH during early gonadal development has not been demonstrated to date in many teleost species, and low circulating levels of LH have been reported in several species during this period.

The initial phases of gametogenesis in females—oogonial proliferation and primary oocyte growth—do not appear to be controlled by pituitary hormones, whereas FSH has been shown to stimulate spermatogonial proliferation in male salmonid fishes. Gonadotropins, however, clearly have critical functions during subsequent stages of gamete growth and maturation in both males and females. During the secondary oocyte growth phase, the gonadotropins stimulate the synthesis of 17β-estradiol in the granulosa cells and its precursor, testosterone, in the thecal cells and their subsequent secretion into the circulation. A major function of 17β-estradiol in females is to regulate the hepatic production of vitellogenins, the egg yolk precursor proteins. Estrogen also regulates the synthesis of vitelline envelope (zona radiata) proteins in the livers of many teleost species. Vitellogenins are rapidly incorporated into the growing oocytes, whose diameters increase dramatically during this prolonged period of oocyte and ovarian growth. The vitellogenins are cleaved into the major yolk proteins lipovitellin and phosvitin, as well as a nonlipoidal derived protein, the β^1-component, whose function is currently unknown (Hiramatsu et al., 2002). The physiological significance of the high circulating levels of testosterone in females during this period is less clear. Testosterone is a precursor for estradiol synthesis, and there is evidence that it participates in the feedback control of gonadotropin secretion by aromatization to estradiol. Other ovarian hormones, such as inhibin and activin, and growth factors, such as insulin-like growth factors

(IGFs), are likely involved in intraovarian regulation of steroidogenesis and follicular growth, as well as pituitary feedback of gonadotropin secretion, although their precise functions in teleosts have not been elucidated.

Spermatogenesis can be divided into three major phases: mitotic proliferation of the spermatogonia from stem cells, meiosis of spermatocytes, and the transformation of spermatids into flagellated spermatozoa (spermiogenesis) (Schulz and Miura, 2002). Sertoli cells are in close contact with the germ cells and provide nutrients and stimuli such as growth factors that regulate their development. The function of Sertoli cells is in turn regulated by FSH and steroid hormones secreted by Leydig cells. Leydig cells produce testosterone and 11-ketotestosterone, as well as trace amounts of 17β-estradiol during spermatogenesis. Androgens regulate all stages of spermatogenesis, probably by controlling the production of IGFs and activin B by Sertoli cells (Schulz and Miura, 2002). Regulation of androgen production is largely under the control of LH when the germ cells are undergoing meiosis. The identification of a nuclear estrogen receptor in croaker testes suggests that estrogens have important functions in the gonads of male fish (Loomis and Thomas, 1999). One function of estrogens in the testis may be to stimulate stem cell renewal divisions (Schulz and Miura, 2002).

The final stages of gamete maturation and release in teleosts are controlled by LH and involve the synthesis of maturation-inducing steroids (MISs), C-21 progestins (Miura et al., 1992; Nagahama, 2000; Nagahama et al., 1993; Thomas, 1994). The MISs have been positively identified as 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in amago salmon (Nagahama and Adachi, 1985) and as 17,20β, 21-trihydroxy-4-pregnen-3-one (20β-S) in Atlantic croaker (Trant and Thomas, 1989). The major MIS for salmonids and cyprinids is 17,20β-P (Scott et al., 1987), whereas 20β-S has been shown to be the predominant MIS in sciaenids and some other perciform fishes (Thomas, 1994). In addition, other C-21 progestins and 11-deoxycorticosteroids identified in several flatfishes may function as MISs in these species (Scott et al., 1987). In females, a surge in LH secretion initiates the process of oocyte maturation (OM), comprised of two gonadotropin-dependent stages (Patiño and Thomas, 1990a). The development of oocyte maturational competence (i.e., the ability to respond to the MIS and complete maturation) occurs during the initial MIS-independent phase (Patiño et al., 2001). This includes upregulation of the MIS receptor (Thomas et al., 2001) and the gap junctions and their associated protein, connexin. This phase is followed by the resumption of meiosis, migration of the nucleus (germinal vesicle) to the animal pole (GVM), dissolution of the nucleus (germinal vesicle breakdown [GVBD]), and changes in the ooplasm, including lipid coalescence, hydration, and formation of an oil droplet (in fish that release pelagic eggs) during the MIS-dependent phase (GVBD phase) (Patiño et al., 2001). The MIS induces GVBD via a cell-surface-initiated, nongenomic mechanism by binding to specific membrane progestin receptors (mPRs). Ligand binding to these receptors results in a decrease in cAMP levels that can be blocked by pertussis toxin, an inhibitor of inhibitory G-protein (G_i) activation, indicating that the receptor activates a G_i (Pace and Thomas, 2005; Thomas et al., 2002; Yoshikuni and Nagahama, 1994). The decrease in cAMP levels releases the oocyte from meiotic arrest via a signal transduction pathway that causes the production of maturation-promoting factor (MPF), which consists of two components: a catalytic cdc2 kinase and cyclin B (Nagahama et al., 1993). Ovulation occurs soon after the completion of OM via a genomic steroid mechanism. The MIS activates a nuclear progestin receptor (nPR) in the ovarian follicle wall (Goetz et al., 1991; Pinter and Thomas, 1997, 1999), causing activation of protein kinase C and the synthesis of arachidonic acid and prostaglandins, which in turn cause contractions of smooth muscle in the follicle wall to expel the oocyte (Goetz et al., 1991; Patiño et al., 2003).

Recent studies have shown that MISs cause maturation of spermatozoa by nongenomic and genomic steroid mechanisms, in Atlantic croaker and salmonids, respectively (Schulz and Miura, 2002; Thomas et al., 2004). Additional research will be required to reconcile these two models of sperm activation in teleosts; however, it is probable that both steroid mechanisms may operate in the same species, as both the nuclear and the membrane progestin receptors have been characterized in the testes of spotted seatrout and Atlantic croaker (Pinter and Thomas, 1997; Thomas, 2000c; Thomas et al., 1998, 2005). Increased sperm motility after gonadotropin or progestin treatment has been reported in salmonids, and this is associated with MIS-induced increases in the pH of the seminal fluid by a genomic mechanism, presumably by binding to the nPR (Miura et al., 1992). It has been demonstrated that

MISs can induce sperm hyperactivity in Atlantic croaker and spotted seatrout by binding to specific mPRs on their sperm membranes (Thomas et al., 1998), resulting in rapid increases in intracellular free calcium and cAMP concentrations (Thomas, 2003). This mechanism of sperm activation is homologous to that in mammals where progesterone binds to the sperm mPRs during their passage through the female reproductive tract to induce hyperactivity and the acrosome reaction via alterations in intrasperm calcium and cyclic nucleotide levels (Blackmore et al., 1991; Revelli et al., 1998). Interestingly, gonadotropin and progestin treatment increases testicular and sperm mPR levels in seatrout and croaker, as well as sperm motility and fertilization success (Thomas et al., 1998). The novel membrane progestin receptor (mPRα) recently discovered in seatrout ovaries (Zhu et al., 2003a) has been localized to the midpieces of fish sperm and is the likely mediator of MIS upregulation of sperm motility (Thomas et al., 2006).

Neuroendocrine Control of Gonadotropin Secretion

The neuroendocrine system controlling LH and FSH secretion in teleosts is complicated, with multiple neural pathways and complex interactions among a broad range of regulatory factors, including neurotransmitters, neuropeptides, amino acids, and steroids (Blasquez et al., 1998; Kah et al., 2000). These factors influence gonadotropin secretion by their actions on neurons that synthesize and release GnRH in the preoptic area and anterior and medial–basal hypothalamic regions or by acting directly on GnRH nerve terminals on gonadotropin-producing cells in the anterior pituitary. The neuroendocrine system of teleost fish differs from that of tetrapods in that it lacks a neurosecretory organ (median eminence) at the base of the hypothalamus for the secretion of releasing hormones and a portal system to deliver them to the pituitary cells. Instead, the anterior pituitary is directly innervated by both stimulatory and inhibitory pathways that originate in the preoptic area and hypophysiotropic regions of the hypothalamus and terminate in the vicinity of the hormone-producing cells.

Multiple forms of GnRH have been discovered in different brain regions of teleost fish (Gothilf et al., 1996; Mohamed et al., 2005). The form of GnRH released from the nerve terminals in the pituitary is the major stimulatory influence on gonadotropin secretion. Sea bream GnRH is the primary gonadotropin-releasing hormone in several perciform fishes, whereas salmon GnRH performs this function in salmonid and some cyprinid fishes (Lethimonier et al., 2004). GnRH binds to specific G-protein-coupled receptors (GPCRs) on the gonadotropes that can couple to multiple G-proteins ($G_{q/11}$, G_s, and G_o), resulting in activation of calcium-/protein kinase C (PKC)-dependent and other pathways to stimulate gonadotropin secretion (Ando et al., 2001). Hypothalamic and pituitary GnRH content increases during the reproductive cycle (Holland et al., 1998; Khan et al., 2001). GnRH regulates the number of GnRH receptors on the gonadotropes which in turn modulate the responsiveness of the cells to subsequent GnRH stimulation. GnRH receptor content increases during the later phases of the reproductive cycle to potentiate the stimulatory action of GnRH, coincident with other changes in the neuroendocrine system, to achieve a preovulatory surge in LH secretion (Habibi et al., 1989). The secretion of LH is highly regulated, whereas FSH secretion in the few salmonid species investigated to date appears to be largely constitutive, paralleling changes in FSH mRNA and protein levels (Swanson et al., 2003). The monoamine neurotransmitter serotonin (5-HT) can act on the GnRH system in the preoptic–anterior hypothalamic area (POAH) and also potentiate the action of GnRH on LH secretion in Atlantic croaker and goldfish (Khan and Thomas, 1992; Somoza and Peter, 1991; Somoza et al., 1988) at certain times of the day and phases of the reproductive cycle (Khan and Thomas, 1994). Dopamine exerts a strong inhibitory control of LH secretion in carps and some catfishes (Blasquez et al., 1998), whereas it has a minor influence in salmonids and has no inhibitory effect on LH secretion in Atlantic croaker and some other perciform fishes (Copeland and Thomas, 1989). Several other neuromeodulatory factors influence LH secretion in teleosts. GABA stimulates LH secretion in goldfish and regressed Atlantic croaker and inhibits gonadotropin secretion in croaker with fully developed gonads (Khan and Thomas, 1999; Trudeau and Peter, 1995). The negative feedback effect of gonadal steroids (estradiol, testosterone) also varies during the reproductive cycle, as it is stimulatory on GnRH-induced LH secretion in immature and early recrudescing individuals and inhibitory in fish at the end of the reproductive cycle (Khan et al., 1999; Mathews et al., 2002; Trudeau and Peter, 1995).

Actions of Hormones at Target Tissues

Activation of Intracellular Second Messengers

Binding of peptide, protein, and steroid hormones to cell-surface receptors on target cells activates intracellular signal transduction systems, leading to the hormonal response. Binding of GnRH to a specific GPCR on gonadotropes activates several signal transduction pathways in the three teleost models investigated in detail: goldfish, salmonids, and tilapia (Ando et al., 2001; Chang and Jobin, 1994; Yaron et al., 2001). GnRH causes rapid activation of a G-protein ($G_{q/11}$) and a second-messenger cascade involving phospholipase C (PLC) and the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG causes activation of protein kinase C (PKC), whereas IP_3 has been shown to cause intracellular calcium concentrations to increase in mammalian gonadotropes due to mobilization from internal stores. GnRH also induces calcium influx through voltage-sensitive calcium channels (VSCCs) into fish gonadotropes. The increase in intracellular calcium concentrations seems to be of primary importance in the stimulation of gonadotropin secretion, whereas both calcium levels and activation PKC are involved in GnRH regulation of gonadotropin subunit gene expression (Ando et al., 2001; Gur et al., 2001). Moreover, recent evidence suggests that PKC acts via stimulation of mitogen-activated protein kinase (MAPkinase). In addition, phospholipase A_2 and arachidonic acid appear to be involved in gonadotropin release from tilapia pituitaries. Also, a cAMP-dependent protein, protein kinase A (PKA), is activated by GnRH in tilapia (Yaron et al., 2001). The signal transduction systems activated by gonadotropins are highly conserved among the vertebrates. The adenylate cyclase system is of central importance in mediating the steroidogenic actions of gonadotropins in fish as well as in other vertebrates (Van Der Kraak and Wade, 1994). Cyclic AMP levels increase markedly in spotted seatrout ovaries within 15 minutes of gonadotropin stimulation *in vitro*, prior to the increase in steroid synthesis (Singh and Thomas, 1993). Calcium from both intracellular and extracellular pools is also an important component of the signaling pathway (Benninghoff and Thomas, 2005). The calcium-binding protein calmodulin participates in the regulation of intracellular calcium levels, whereas VSCCs regulate calcium influx. Other second messengers such as PKC, inositol-1,4,5-triphosphate, diacylglycerol, and arachidonic acid appear to be components of the gonadotropin signaling pathway in certain steroidogenic tissues (Van der Kraak and Wade, 1994). Therefore, chemicals could potentially interfere with a variety of signal transduction systems to modulate the actions of gonadotropin.

Genomic Steroid Actions via Nuclear Steroid Receptors

The classic genomic mechanism of steroid action involves diffusion of steroids into the cell where they bind to intracellular receptors (nuclear steroid receptors), resulting in receptor activation and binding to hormone response elements on genes (in conjunction with other transactivating proteins) to alter their rates of transcription (Figure 10.2) (Beato and Sanchez-Pacheco, 1996; Tsai and O'Malley, 1994). The nuclear steroid receptors—estrogen (ER), androgen (AR), progestogen (PR), glucocorticoid (GR), and mineralocorticoid (MR)—are members of the nuclear receptor superfamily of ligand-activated transcription factors that includes receptors for thyroid hormone, vitamin D, ecdysone, and retinoids as well as many orphan receptors for which the ligands are currently unknown (Laudet, 1997). Nuclear steroid receptor proteins have a similar modular structure with six functional domains (A to F). At the *N*-terminal end, the hypervariable A/B domains possess sequences required for transcriptional activation. The DNA-binding region (C domain) is the most highly conserved region of the receptor protein (60 to 95% homology) and has two protruding zinc fingers that enable the ligand-activated receptor to bind to hormone response elements (specific DNA sequences in the regulatory regions of genes) of the target genes. The variable hinge region (D domain) is involved in nuclear localization. The large ligand-binding domain (LBD), or E domain, is conserved and displays a high degree of sequence homology, particularly with members of the same receptor subfamily (38 to 60% sequence identity between teleostean and mammalian ERs) (Hawkins et al., 2000). In addition, the LBD is involved in receptor dimerization, heat shock protein binding, and transcriptional activation. At the *C*-terminal end, the F domain is highly variable and of unknown function.

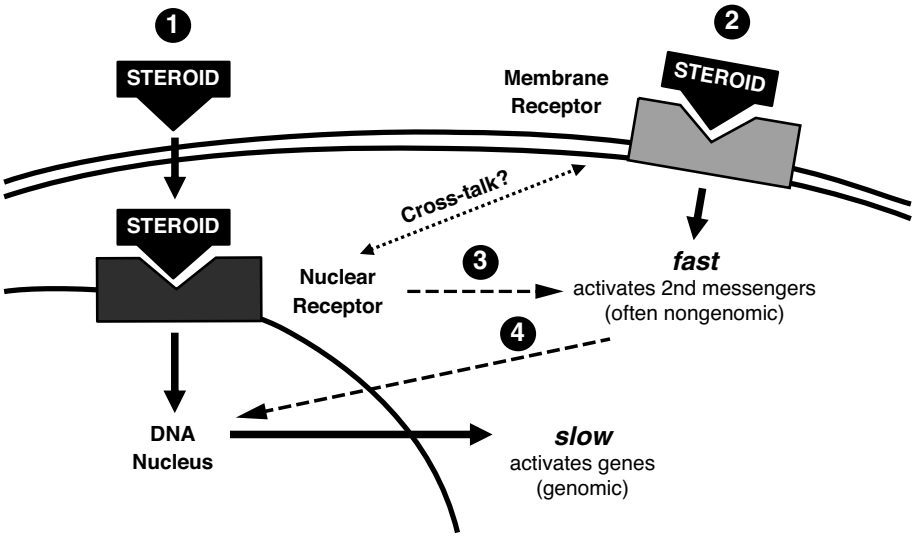

FIGURE 10.2 Major receptor-mediated mechanisms of steroid action at target cells. (1) Classic mechanism of steroid action via binding to intracellular nuclear steroid receptors, translocation to the nucleus, and binding to hormone response elements of genes resulting in alterations in their rates of transcription (genomic mechanism). This mechanism, involving new mRNA and protein synthesis, is relatively slow and occurs over a timeframe of hours to days. (2) Alternative mechanism of steroid action via binding to receptors on the cell surface, called membrane receptors, resulting in activation of ion channels or intracellular second messengers. This mechanism initiates a rapid hormonal response within a few minutes that is often nongenomic. The identities of most membrane steroid receptors are unresolved. Some membrane receptors are nuclear-receptor-like; others, such as the ovarian progestin membrane receptor, are novel proteins unrelated to the nuclear steroid receptors. Cross-talk is also possible between these two signaling pathways. Additional mechanisms of steroid action that have been identified involve (3) activation of intracellular second messengers through the intracellular nuclear receptor and (4) alterations in gene transcription via membrane steroid receptors. (From Thomas, P.C. et al., in *The Fish Oocyte: From Basic Studies to Biotechnological Applications*, Babin, P.J. et al., Eds., Springer, Dordrecht, 2007, pp. 203–234. With permission.)

Nuclear steroid receptors are localized primarily in the nucleus (ER) or cytoplasm (GR) prior to ligand binding and are associated with heat shock proteins. Steroid binding causes a conformational change of the receptor, resulting in its activation which is associated with dissociation of heat shock proteins, phosphorylation of specific amino acids, and dimerization. The activated receptor is translocated to the nucleus and binds to DNA via the DNA-binding domain (C domain). The two zinc fingers in the C domain orientate the receptor dimers to enable binding of the intervening sequences to the hormone response elements on DNA. The hormone response elements consist of two half sites, each one binding one receptor molecule of the dimer. The number, spacing, and structure of hormone response elements modulate the affinity and specificity of steroid receptor binding to genes and are thought to be critical regulators of steroid hormone action. Once the receptor is aligned on the gene, its two transcription activation domains activate transcription factors (TFBIIB and TFBIID) to signal assembly of other transcription factors and DNA polymerase II at the transcription start site (TATA box) to initiate transcription. In addition to gene activation, transcriptional repression by steroid hormones frequently occurs as a result of inhibition of enhancer elements such as AP-1 or by interactions with corepressors.

Nuclear Steroid Receptors in Fish

The responsiveness of target tissues to steroid hormones is largely dependent on their expression of steroid hormone receptors, highly responsive target tissues having elevated receptor concentrations in comparison to other tissues. Nuclear steroid receptor expression is regulated by steroid hormones and therefore fluctuates in response to alterations in steroidogenesis; for example, the nuclear estrogen receptor (nER), which is regulated by estrogens in teleosts, demonstrates a marked seasonal cycle in the livers of spotted seatrout that parallels the changes in circulating 17β-estradiol levels during the period of vitellogenesis in this species (Smith and Thomas, 1991). Similarly, nuclear androgen receptor (nAR) levels are regulated

by androgens in teleosts, and gonadal concentrations of the receptor increase during the reproductive season (Larsson et al., 2002; Sperry and Thomas, 1999a). Target tissue responsiveness in mammals and probably in teleosts to estrogens and several other steroid hormones is also dependent on which nuclear receptor subtypes are expressed. Two distinct nuclear ER subtypes (ERα and ERβ) are present in mammals and three ERs (ERα, ERβa and ERβb) in advanced teleosts with distinct but overlapping tissue distributions and different steroid-binding affinities (Hawkins and Thomas, 2004; Hawkins et al., 2000; Kuiper et al., 1997). Multiple ARs with different tissue distributions and ligand-binding properties have also been identified in teleosts (Ikeuchi et al., 2001; Sperry and Thomas, 1999a,b, 2000). This multiplicity in nuclear receptor subtypes modulates not only tissue responsiveness to natural steroid hormones but also interactions with xenobiotic chemicals, a topic that is discussed later in this chapter.

Nongenomic Steroid Actions via Membrane Steroid Receptors

In addition to the classic mechanism of steroid action, extensive evidence now suggests that steroids also exert rapid, nongenomic actions by binding to receptors on the surface of target cells and activating signal transduction pathways, leading to a biological response (Figure 10.2) (Falkenstein et al., 2000; Norman et al., 2004; Revelli et al., 1998; Watson and Gametchu, 1999). Progesterone treatment, for example, causes a marked increase in intracellular calcium levels in mammalian sperm in less than a minute, resulting in the acrosome reaction and hypermotility (Blackmore et al., 1991). Specific membrane receptors and rapid nongenomic actions for estrogens, androgens, and progestins have recently been identified throughout the reproductive system in vertebrates, including the hypothalamus, pituitary, gonads, gametes, steroidogenic cells, and primary and secondary reproductive structures such as the breast (Revelli et al., 1998). Nongenomic steroid actions have been shown to have important functions in several reproductive processes such as the activation of sperm (Blackmore et al., 1991; Revelli et al., 1998) in mammals, but their precise physiological roles in many other reproductive tissues remain unclear.

Membrane Steroid Receptors in Fish

Membrane receptors for all the sex steroids have been identified in teleosts (Thomas, 2000c, 2003). A high-affinity membrane estrogen receptor (mER) has been characterized in Atlantic croaker testicular membranes and is the likely intermediary in a rapid, cell-surface-initiated, nongenomic action of estrogens to downregulate testicular androgen production in this species (Loomis and Thomas, 2000). The mER is also present in croaker ovaries and activates a stimulatory G-protein (G_s), resulting in increased cAMP production (Thomas et al., 2003). Androgens also can regulate gonadal steroidogenesis in Atlantic croaker, causing downregulation of ovarian estrogen biosynthesis via a nongenomic mechanism (Braun and Thomas, 2003). A membrane androgen receptor has been identified in croaker ovaries and fully characterized in this species (Braun and Thomas, 2004). Probably the most intensively studied and best characterized model of a cell-surface-initiated, nongenomic steroid action is the induction of oocyte maturation (OM) in teleosts and amphibians by progestin MISs (Nagahama et al., 1994; Thomas, 1994; Thomas et al., 2002). The mPRs on oocytes thought to mediate these actions of the fish MISs, 17,20β-P and 20β-S, have been identified and fully characterized in several teleost species (Patiño and Thomas, 1990b; Thomas et al., 2002). MIS receptor concentrations are upregulated by gonadotropin during an early stage of oocyte maturation, and this is associated with the oocytes becoming responsive to the MISs and the completion of OM after hormone treatment (Thomas et al., 2001). In addition, an mPR has been characterized on spotted seatrout sperm and is the likely intermediary in 20β-S stimulation of sperm motility via increases in intracellular calcium and cAMP levels, resulting in increased fertilization success in this species (Thomas, 2003; Thomas et al., 1998).

Recently, a novel gene was cloned and sequenced from a spotted seatrout cDNA ovarian library that has all the characteristics of the mPR mediating MIS regulation of oocyte maturation in this species (Thomas et al., 2002; Zhu et al., 2003a). Subsequently, 13 additional closely related cDNAs were identified in other vertebrate species, including 3 in humans that represent 3 distinct clades and also have characteristics of mPRs (Zhu et al., 2003b). These mPRs are not structurally related to nuclear steroid receptors, but instead have 7 transmembrane domains, which is a characteristic of G-protein-coupled receptors. Recent studies show the mPR in spotted seatrout is coupled to an inhibitory G-protein

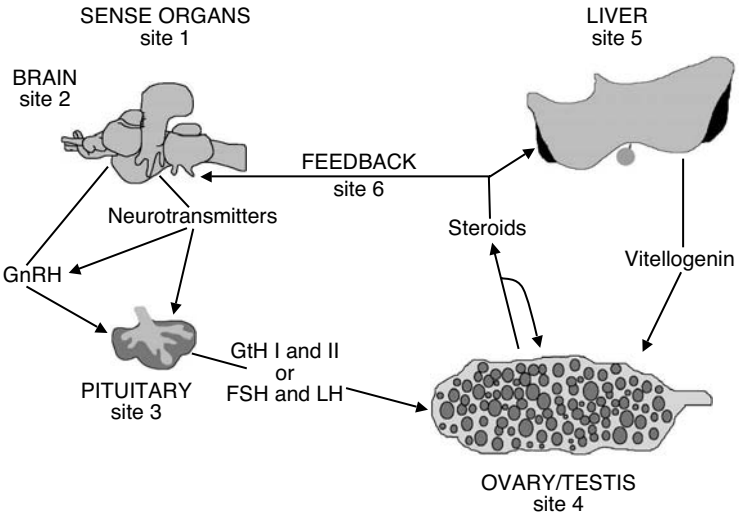

FIGURE 10.3 Potential sites of chemical interference with reproductive endocrine function in teleosts along the hypothalamic–pituitary–gonadal axis. (Site 1) Sense organs—eyes, pineal, olfactory organs, etc. (Site 2) Brain—hypothalamic regions, particularly the hypophysiotropic region of the hypothalamus; causing alterations in neurotransmitters and neuropeptides, especially GnRH. (Site 3) Pituitary—gonadotropes, GnRH, and neurotransmitter nerve terminals and their receptors; altering GnRH and neurotransmitter release and GnRH receptor concentrations and resulting in changes in LH and FSH secretion. (Site 4) Gonads—germ cells, gametes, steroidogenic cells, etc. and associated steroid receptors; resulting in altered gametogenesis, gamete maturation, steroid and peptide hormone secretion, and steroid action. (Site 5) Liver—cytochrome P-450 and conjugating enzymes; causing changes in steroid metabolism, clearance, and circulating steroid hormone titers; estrogen nuclear receptors causing changes in the production of vitellogenin and zona radiata proteins in both sexes. (Site 6) Hypothalamus and pituitary—sex steroid and gonadal peptide hormone receptors and steroidogenic enzymes; causing alterations in feedback control of neurotransmitter, as well as GnRH, LH, and FSH synthesis and secretion in the pituitary. Sex steroids also act within the gonads to exert feedback control of steroidogenesis. The steroids 17β-estradiol and testosterone are present in both sexes, although 17β-estradiol is usually low in males; 11-ketotestosterone is a male-specific androgen in the majority of species; 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) are maturation-inducing steroids (MISs) in both males and females. Other C-21 progestins may be MISs in a few species. *Abbreviations:* GnRH, gonadotropin-releasing hormone; FSH or GTH I, follicle-stimulating hormone; LH or GTH II, luteinizing hormone. (From Thomas, P. and Khan, I.A., in *Chemically Induced Alterations in Functional Development and Reproduction of Fishes*, Rolland, R. M. et al., Eds., Society of Environmental Toxicology and Chemistry, Pensacola, FL, 1997, pp. 29–51. With permission.)

and that progestin treatment causes a downregulation of adenylyl cyclase activity, resulting in a decrease in cAMP levels and release of the oocyte from meiotic arrest (Pace and Thomas, 2005; Thomas, 2003; Thomas et al., 2002). Activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway by the MIS is also required for OM in Atlantic croaker and striped bass (Pace and Thomas, 2006; Weber and Sullivan, 2001). Direct evidence for an involvement of the mPR in MIS induction of OM in zebrafish and goldfish has been obtained by blocking OM by downregulating the receptor through microinjection of oocytes with mPR antisense oligonucleotides (Tokumoto et al., 2006; Zhu et al., 2003a). The discovery of this new family of steroid receptors provides the first plausible explanation of how steroids can act on the cell surface to cause rapid, nongenomic hormonal responses in target cells.

Sites of Chemical Interference on the Hypothalamic–Pituitary–Gonadal Axis

Chemicals can act on multiple targets in the endocrine system and by a variety of mechanisms to disrupt endocrine function. Figure 10.3 shows some potential sites of chemical action on the hypothalamic–pituitary–gonadal (HPG) axis that result in interference with reproductive endocrine function. Chemicals could be detected as noxious by sense organs such as the eyes and olfactory epithelium or could interfere

with sex pheromone action by binding to their olfactory receptors, resulting in activation of inhibitory pathways controlling neuroendocrine function and reproductive behaviors (site 1) (Moore and Lower, 2001). Alternatively, the chemicals could act in the hypothalamus to influence neurotransmitter function controlling GnRH secretion or directly influence GnRH neurons (site 2) (Khan and Thomas, 2001). Changes in the secretion of GnRH and regulatory neurotransmitters would secondarily affect pituitary GnRH receptor levels and gonadotropin secretion from the pituitary in response to GnRH. In addition, certain chemicals can act directly at the pituitary to influence GnRH signaling pathways in the gonadotrope and gonadotropin secretion (site 3) (Thomas, 1993). These chemically induced changes in gonadotropin secretion often result in alterations in gonadal function and development, especially steroid hormone production and gamete maturation. Some chemicals exert their actions directly on the gonads to disrupt endocrine function by influencing the activities of gonadotropin second-messenger pathways and enzymes involved in the synthesis of steroid hormones (site 4) (Ankley et al., 2005; Benninghoff and Thomas, 2005; Freeman and Idler, 1974; Thomas and Khan, 1997; Van Der Kraak et al., 1992). Alterations in steroid production can dramatically influence steroid levels in the blood and subsequently steroid actions at their target tissues such as the gonads, accessory reproductive tissues, liver, and neuroendocrine system. Increases in the metabolic clearance rate of steroids due to induction of hepatic cytochrome P450 enzymes by certain chemicals such as polychlorinated biphenyls (PCBs) can also influence circulating steroid levels (site 5) (Sivarajah et al., 1978; Yano and Matsuyama, 1986). Steroid hormone action can also be affected at these target tissues by direct interactions of chemicals with both nuclear and membrane steroid receptors, resulting in agonism or antagonism of steroid hormone action; for example, the steroid feedback system that influences reproductive neuroendocrine function (site 6) could be affected by alterations in the production (Kishida et al., 2001), clearance, or action of steroids (Harris et al., 2001; Khan and Thomas, 1998). Similarly, the stimulation of vitellogenesis by chemicals could be due to their direct interactions with the estrogen receptor in the liver, which regulates vitellogenin production (site 5) (Jobling and Sumpter, 1993; Thomas and Khan, 1997); could occur indirectly due to actions at other levels of the HPG axis that result in alterations in blood estradiol levels; or could be due to several mechanisms acting simultaneously. For some chemicals, actions at multiple sites via several mechanisms have been demonstrated (Van Der Kraak et al., 1992).

Mechanisms of Endocrine Toxicity

The main features of the endocrine system, discussed in the previous sections, involves the secretion of hormones into the circulation, their binding to specific receptors on target tissues, and the subsequent intracellular hormonal responses. In general, the magnitude of the cellular response to hormones is dependent on the number of receptors occupied by the hormone which, in turn, is related to the concentration of free (or loosely bound) hormone in the circulation. Xenobiotic chemicals, therefore, can potentially influence endocrine function by affecting hormone levels in the blood via alterations in hormone secretion or metabolic clearance or by interfering with hormone action at the receptor or at other sites along the hormone signal transduction pathway. Evidence has been obtained for all these types of endocrine disruption in fishes.

Alterations of Hormone Secretion

Neuroendocrine Secretion

A large body of evidence, mostly circumstantial, indicates that many compounds, including lead, mercury, organochlorine and organophosphorus pesticides, PCB mixtures, and mimics or antagonists of steroid hormone action impair reproductive endocrine function (i.e., endocrine-disrupting chemicals [EDCs] that act on the reproductive system) at the hypothalamic level in mammals, fish, and other vertebrates (Cooper et al., 1999; Desaulniers et al., 1999; Katti and Sathyaneson, 1986; Khan and Thomas, 1997; Thomas and Khan, 1997). Investigations of the neuroendocrine targets of EDCs, however, are complicated by the multiplicity and complexity of neuroendocrine pathways that control reproduction and the

wide variety of potential chemical interactions; consequently, the sites and mechanisms of neuroendocrine toxicity for the majority of EDCs remain poorly understood.

Alterations of Gonadotropin Secretion via Changes in Hypothalamic Monoamine Metabolism. Chemicals that alter neurotransmitter concentrations, such as organochlorine pesticides, probably influence neuroendocrine function and ultimately reproduction, although there are only a few reports on this potentially important mechanism of endocrine disruption (Cooper et al., 1999; Goldman et al., 1990; Khan and Thomas, 1997). The strongest evidence to date for this mechanism of neuroendocrine toxicity has been obtained in the Atlantic croaker exposed to a PCB mixture, Arochlor® 1254. The marked impairment of LH secretion observed in croaker after exposure to Arochlor® 1254 provided initial evidence that the neuroendocrine system is a major target of PCBs in teleosts (Thomas, 1989). Subsequently, it was shown that disruption of LH secretion occurs in croaker with tissue PCB levels (13 ppm in brain) comparable to those in fish from heavily impacted environments and that this impairment was accompanied by alterations in brain neurotransmitter levels (Khan and Thomas, 1997). Serotonin concentrations in the hypothalamus were significantly decreased (30 to 35%) after 30-day exposures to the PCB (0.1 mg per 100 g b.w. per day) in the diet, whereas tissue levels of its metabolite, 5-hydroxyindolacetic acid, showed only minor changes. Serotonin has been shown to exert a stimulatory influence on LH secretion in croaker and other vertebrates (Khan and Thomas, 1992). A subsequent study showed that the PCB-induced decline in hypothalamic 5-HT concentrations was due to inhibition of tryptophan hydroxylase, the rate-limiting enzyme in serotonin synthesis (Khan and Thomas, 2001). The decreased 5-HT activity after PCB exposure resulted in decreased hypothalamic concentrations of GnRH and its secretion, leading to downregulation of GnRH receptors on gonadotropes and a decreased LH response to GnRH stimulation. This loss of responsiveness to GnRH and downregulation of GnRH receptors after Arochlor® 1254 treatment could be restored with an implant of luteinizing-hormone-releasing hormone (LHRH) (Khan and Thomas, 2001). Moreover, a specific inhibitor of tryptophan hydroxylase, *para*-chlorophenylanaline, mimicked these neuroendocrine effects of the PCB mixture as well as the subsequent reproductive impairment, whereas cotreatment of the PCB-dosed fish with 5-hydroxytryptophan, which bypasses this biosynthetic step, reversed the PCB effects (Khan and Thomas, 2001; Thomas and Khan, 2004). Recent studies indicate that the decrease in tryptophan hydroxylase activity is due to a decline in hypothalamic content of the protein, which appears to be related to oxidant damage, because the PCB effects can be reversed by supplementation with an antioxidant (vitamin E) in the diet (Khan and Thomas, 2004). Mammalian studies also suggest that the enzyme is susceptible to damage by reactive oxygen species, and vitamin E has a protective effect (Adachi et al, 1989; Cash, 1998). Neuroendocrine disruption associated with alterations of neurotransmitter function has also been reported in croaker after exposure to lead (Thomas and Khan, 1997). Interestingly, both Arochlor® 1254 and lead have been shown to cause increased lipid peroxidation in croaker brain and ovarian tissues in addition to altering their antioxidant status (Thomas and Juedes, 1992; Thomas and Wofford, 1984, 1993).

Gonadal Steroidogenesis

A range of chemicals have been shown to exert direct effects on gonadal steroidogenesis in teleosts via several mechanisms. Several polycyclic aromatic hydrocarbons (naphthalene, β-naphthoflavone, and retene) were shown to potentiate the stimulatory effects of gonadotropin on testicular androgen production but did not alter basal secretion (Evanson and Van Der Kraak, 2001). The site of β-naphthoflavone action appeared to be independent of calcium and to be mediated by increases in cAMP, which suggests interference with gonadotropin action (see subsequent section). In contrast, the stimulatory effects of Arochlor® 1254 on gonadotropin-stimulated ovarian estrogen production were attenuated by treatment with inhibitors of calmodulin and voltage-sensitive calcium channels, indicating a role for intracellular calcium in the stimulatory effects of the PCB mixture (Benninghoff and Thomas, 2005). The reduction in the steroidogenic capacity of gonads of freshwater fishes observed after exposure to the phytosterol β-sitosterol, a component of bleached pulp mill effluent (MacLatchy and Van der Kraak, 1995), was associated with a decrease in the mitochondrial pool of cholesterol (Leusch and MacLatchy, 2003). Peroxidation of lipids and proteins by chemicals such as PCBs, including the steroidogenic enzymes

themselves, is another potential mechanism by which steroid synthesis could be altered, although clear evidence for this mechanism has only been obtained in fish interrenal tissues (see subsequent section on HPI axis).

Interference with Hormone Actions

Intracellular Second Messengers

A well-known toxic action of heavy metals such as cadmium and lead, as well as organic compounds such as dichlorodiphenyltrichloroethane (DDT) and PCBs, is interference with calcium homeostasis (Inglefield and Shafer, 2000; Pounds, 1990). Some of these chemicals appear to alter calcium signaling in endocrine tissues, resulting in altered hormone secretion (Thomas, 1999). Chronic treatment with cadmium was found to advance gonadal recrudescence and increase the secretion of gonadotropin (LH) and sex steroids in Atlantic croaker (Thomas, 1989). It was later found that the metal can directly stimulate the secretion of these hormones from pituitary and ovarian tissues in *in vitro* incubations (Thomas, 1993). Perifusion of 50-μ*M* cadmium was shown to markedly increase basal gonadotropin secretion and restore the gonadotropin response to GnRH in calcium-free media. Moreover, the spontaneous secretion of LH in response to cadmium in the pituitary perifusion system was blocked by prior treatment with verapamil, an inhibitor of voltage-sensitive calcium channels (VSCCs), and calcium influx into the gonadotrop, which suggests that the metal activates the calcium-dependent signaling pathway normally induced by GnRH via its actions on VSCCs (Thomas, 1999). Heavy metals have also been shown to influence cAMP concentrations and adenylyl cyclase activities in endocrine tissues, possibly by alterations in calcium or zinc homeostasis or calmodulin activation, resulting in altered enzyme activities (Singhal, 1981). At the gonadal level cadmium caused a concentration-dependent increase in 17β-estradiol secretion and cAMP levels and caused significant increases in androgen and estrogen secretion at the lowest concentration tested (0.01 ppm) (Thomas and Khan, 1997). Interestingly, direct stimulatory effects of cadmium on gonadotropin secretion and gonadal steroidogenesis also have been demonstrated in mammalian tissues (Cooper et al., 1987; Laskey and Phelps, 1991).

Interference with Genomic Steroid Actions via Nuclear Steroid Receptors

Most of the evidence for chemical interference with endocrine functions in vertebrates has been obtained for genomic steroid actions via interactions with nuclear steroid receptors, especially the nuclear ER. The occurrence of significant environmental concentrations of estrogens capable of eliciting estrogenic responses was initially detected by the measurement of high levels of vitellogenin in the blood of male and juvenile fish exposed to sewage treatment works (STW) discharge water in several rivers in England (Jobling and Sumpter, 1993; Purdom et al., 1994) and has since been reported in male fish collected from many freshwater and estuarine sites in Europe, Japan, and North America (Allen et al., 1999; Folmar et al., 1996; Hashimoto et al., 2000; Larsson et al., 1999). Normally, plasma levels of this yolk precursor protein are very low or are undetectable in male and juvenile fish; however, circulating vitellogenin levels in the milligram/milliliter range have been detected in male and juvenile rainbow trout exposed to STW discharge water (Purdom et al., 1994; Larsson et al., 1999).

Numerous laboratory and field studies over the past 15 years have demonstrated that a broad range of environmental chemicals with a variety of structures can elicit estrogenic responses in fish and other vertebrates via binding to nuclear ERs (Figure 10.4). Activation of nuclear ERs by these chemicals increases the transcription of estrogen-responsive genes such as vitellogenin and zona radiata proteins and nuclear ERs, as well as a large number of genes detected on microarrays whose functions are currently unknown (Denslow et al., 1999), resulting in disruption of gonadal sex differentiation and reproductive processes such as secondary oocyte growth and testicular function (Bulger and Kupfer, 1985; Jobling and Sumpter, 1993). The structures of several estrogenic chemicals are shown in Figure 10.5. Many estrogenic xenobiotic chemicals (xenoestrogens) that bind to mammalian ERs show similar binding affinities for teleost ERs. Xenoestrogens such as *ortho,para* derivatives of DDT, nonylphenol, bisphenol A, hydroxylated metabolites of several PCB congeners, and kepone have relative binding

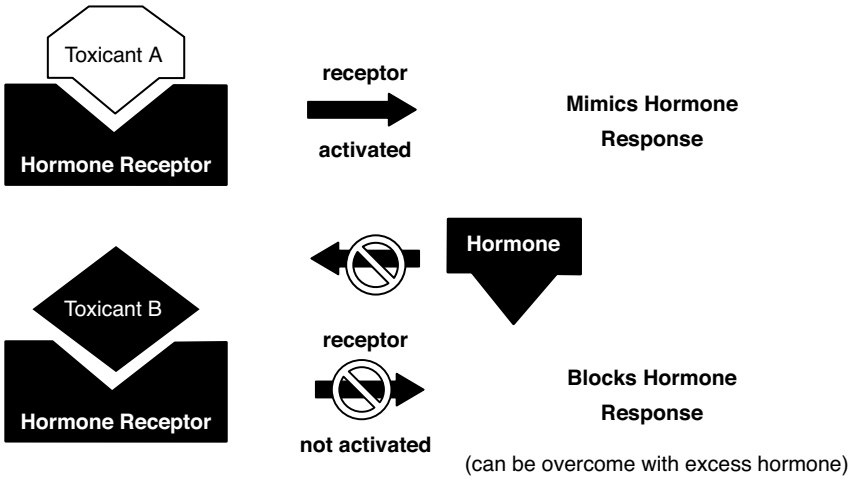

FIGURE 10.4 Possible consequences of competitive binding of a toxicant to a hormone receptor. Binding of toxicant A causes activation of the receptor, mimicking the hormone action (agonist). Binding of toxicant B does not activate the receptor or causes weak activation, resulting in either no hormonal response or blocking hormone action when the hormone is present (antagonist). Increasing the hormone concentrations will displace some or all of the toxicant from the binding site, thereby reducing its antagonistic action. (Adapted from McLachlan, J.A., *Environ. Health Perspect.*, 101, 386–388, 1993.)

affinities for teleost ERs 10^{-3} to 10^{-4} times lower than that of the natural ligand 17β-estradiol, similar to the relative binding affinities for mammalian ERs (Loomis and Thomas, 1999; Nelson, 1974). Thus, the majority of xenoestrogens are weak agonists, and their potency is due to their accumulation and persistence in biota.

In contrast, the majority of environmental contaminants that interact with nuclear androgen and progesterone receptors antagonize the actions of androgens and progestins (Gray et al., 1996, 2004; Kelce et al., 1995; Laws et al., 1995; Lundolm, 1991). Chemicals with antiandrogenic activities mediated via the nuclear AR in mammals include *para,para* derivatives of DDE and DDT, the dicarboximide fungicides vinclozolin and procymidine and their metabolites, the conazole fungicide prochloraz, and the polybrominated biphenyl mixtures DE-71 and DE-100 (Gray et al., 2004). Phthalate esters, which possess some antiandrogenic properties, do not bind to the nuclear AR. Recently, chemicals with androgenic activities that bind to the nuclear AR and cause masculinization of fish have been detected in pulp and paper mill effluents and in feedlot effluents (Gray et al., 2004; Jenkins et al., 2000; Larsson and Forlin, 2002; Larsson et al., 2006; Orlando et al., 2003; Parks et al., 2001). Masculinization effects observed include the development of a gonopodium in female mosquitofish and the development of nuptial tubercles and reduced vitellogenin levels in female fathead minnow (Ankley et al., 2003; Howell et al., 1980).

The androgenic chemicals in these effluents have not been identified to date, although trenbolone, the anabolic steroid administered to domestic animals, or an active metabolite is a likely candidate for a potent androgen in feedlot effluents. Trenbolone has recently been shown to have androgenic effects in the fathead minnow, causing the development of nuptial tubercles in females, an effect blocked by co-administration of the nuclear androgen receptor antagonist flutamide (Ankley et al., 2004). In contrast, metabolites of the fungicide vinclozolin, which are competitors for androgen binding to fish androgen receptors (Sperry and Thomas, 1999) and have antiandrogenic activities, have been shown to downregulate the activity of the aromatase enzyme in fathead minnow (Ankley et al., 2005) and therefore have the potential to impair both androgenic and estrogenic actions in fish. In conclusion, extensive evidence suggests that a broad range of contaminants that influence the endocrine system are present in sufficient concentrations in many polluted environments to alter genomic steroid action mediated by nuclear estrogen, androgen, and progesterone receptors. Factors that affect the degree of endocrine disruption by this mechanism are discussed in the following section, and evidence for its occurrence in natural populations of fish inhabiting polluted waters is reviewed in later chapters.

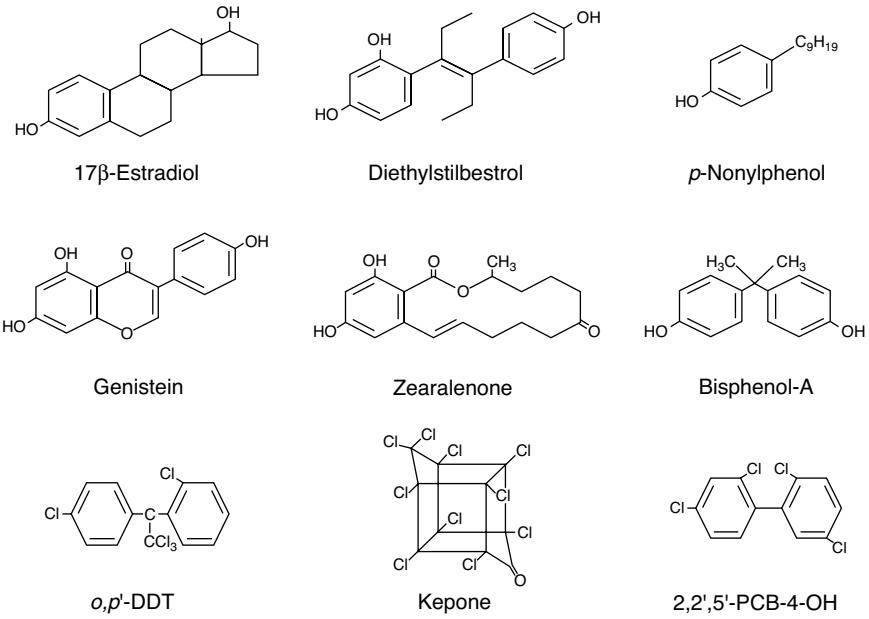

FIGURE 10.5 Structures of some estrogenic chemicals, including the natural hormone 17β-estradiol, the major estrogen in most vertebrates; diethylstilbestrol, a potent synthetic estrogen; genestein, a phytoestrogen; zearanolone, an estrogenic mycotoxin; and a variety of environmental estrogens (xenoestrogens): the organochlorine pesticide *o,p′*-DDT (1,1,1-trichloro-2-(*p*-chlorophenyl)-2(*o*-chlorophenyl)ethane) (the *ortho* and *para* derivatives of DDE and DDD are also xenoestrogens); kepone, an organochlorine pesticide (chlordecone); *p*-nonylphenol, the degradation product of the alkylphenol ethoxylate nonionic surfactants (octylphenol is also a xenoestrogen); bisphenol-A, a plasticizer; and 2,2′,5′-trichloro-4-biphenylol, the hydroxylated metabolite of the PCB 52 congener (other hydroxyl metabolites of PCB congeners such as 2′,4′,6′-trichloro-4-biphenylol and 2′,3′,4′,5′-tetrachloro-4-biphenylol are also xenoestrogens).

Factors That Influence Steroid-Receptor-Mediated Endocrine Disruption

The degree of receptor-mediated endocrine disruption is at least partially dependent on the proportion of the receptors binding the chemical which, in turn, is related to the binding affinity of the chemical for the receptor as well as the chemical and steroid concentrations in the target tissues. The endocrine-disrupting activities of these chemicals, however, can be significantly altered by a variety of other factors. Xenobiotic chemicals can act as agonist or antagonists, depending on the conformational changes to the receptor that they induce upon binding and their subsequent interactions with regulatory proteins in a cell-specific and probably a species-specific manner (Figure 10.3) (McDonnell, 2003).

Species differences in the binding of xenobiotic chemicals to nuclear steroid receptors and their affinities can potentially influence the degree of endocrine disruption and prevent broad extrapolation of results across species. The majority of xenobiotic chemicals (xenoestrogens) that bind to the nuclear estrogen receptor (nER) in mammals also bind to most teleost nERs investigated to date, although their affinities may differ. As an example, *ortho,para* derivatives of DDT bind to the nERs in both the rat and croaker, whereas the *para,para* derivatives do not (Loomis and Thomas, 1999; Nelson, 1994), although marked species differences in the binding affinities of PCBs, hydroxylated PCBs, and PCB mixtures to human, reptilian, and rainbow trout nERs were observed (Mathews et al., 2000). A wide variety of xenobiotic compounds, including DDT derivatives, that bind to the nuclear progesterone receptor (nPR) and antagonize the actions of progesterone in mammals and birds (Gray et al., 1996) do not bind to the nPR in spotted seatrout ovaries at concentrations up to 10^{-4} *M* and therefore are unlikely to interfere with progestin actions mediated by this receptor (Pinter and Thomas, 1997). Thus, it is not possible to predict from xenobiotic binding studies with the nPR in tetrapods whether these chemicals are likely to interfere with progestin action to block ovulation in fishes.

The presence of different receptor subtypes with differing ligand-binding affinities and specificities and different tissues distributions can have a marked effect on the potencies and actions of steroids and xenobiotic chemicals at different target tissues. Multiplicity of receptor subtypes is commonly observed in the nuclear receptor super family; for example, two nER subtypes, ERα and ERβ, with different ligand-binding affinities and tissue distributions, have been identified in mammals. However, two nERβ subtypes with distinct tissue distributions, ERβa and ERβb, have been described in Atlantic croaker and are also present in other teleost fishes (Hawkins et al., 2000; Hawkins and Thomas, 2004). An interesting finding is that the binding affinity of the nER in croaker testes for estrogens and xenoestrogens is 5 to 10 times greater than that of the nER in croaker livers, which suggests that estrogen actions in the testis may be more susceptible to chemical interference than those in the liver (Figure 10.5) (Loomis and Thomas, 1999). It is not known, however, whether these tissue differences in ER binding affinities are due to differential expression of nER subtypes or are a consequence of other tissue-specific factors. Two androgen receptor subtypes have been identified in croaker, kelp bass, eel, and tilapia (Ikeuchi et al.,2001; Sperry and Thomas, 1999a,b). One nAR is localized in the croaker brain and demonstrates specific binding for testosterone but has low affinity for a variety of chemicals that bind to the nAR in mammals and have antiandrogenic activity in mammalian species (Gray et al., 1996; Sperry and Thomas, 1999a,b). In contrast, the other nAR present in gonadal tissues has binding characteristics similar to those of the mammalian nARs and has a broader specificity for androgens and xenobiotic antiandrogens; therefore, androgen actions mediated by binding to the gonadal AR are likely to be susceptible to interference by xenobiotic antiandrogens, whereas those involving the brain nAR are not.

The concentrations of steroids and their receptors, as well as the potencies and actions of steroid hormones, change during the reproductive life cycles of fishes; consequently, both the pattern and degree of endocrine disruption caused by exposure to a xenobiotic endocrine-disrupting chemical will vary, depending on the fish's developmental or reproductive stage. One of the steroid hormone actions that varies markedly during the reproductive cycle of teleosts is the feedback effect of estrogens on gonadotropin secretion. Estrogens and the xenoestrogen *o,p′*-DDT exert positive effects on basal and GnRH-induced LH secretion in croaker at the beginning of gonadal recrudescence, but at the end of the reproductive cycle the influence of estradiol on LH secretion switches to a negative one (Khan and Thomas, 1998; Khan et al., 1999). Presumably, xenoestrogens also have negative feedback effects on LH secretion at this time, although convincing evidence for this is currently lacking.

Recently, it was realized that chemical interactions with nonclassical steroid actions can potentially influence the pattern and degree of endocrine disruption with the discovery that certain xenobiotic chemicals can also interfere with nongenomic steroid actions by binding to membrane steroid receptors (Thomas, 1999, 2000a,b). Most of the evidence for this mechanism of endocrine disruption has been obtained in fish and is reviewed in a subsequent section. In addition, the potential for crosstalk among various ligands, such as estrogens and epidermal growth factor (EGF), transforming growth factor α (TGF-α), and insulin-like growth factor 1 (IGF-1), and between estrogen and thyroid signaling pathways has been demonstrated in mammalian models (Ignar-Trowbridge et al., 1996; Rooney and Guillette, 2000). Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) and related dibenzo-*p*-dioxins, dibenzofurans, and PCBs can induce antiestrogenic effects in mammals indirectly by binding to the aryl hydrocarbon receptor (AhR) (Safe et al., 1991) and subsequently interfering with estrogen receptor binding to DNA response elements (Khara and Saatcioglu, 1996). Recent evidence, however, indicates that AhR ligands can also exert estrogenic effects and that the agonist-activated AhR/Arnt heterodimer associates with estrogen receptors, resulting in recruitment of unliganded estrogen receptor and co-activator p300 to estrogen-responsive gene promoters and subsequent activation of transcription (Ohtake et al., 2003).

Interference with Nongenomic Steroid Actions via Membrane Steroid Receptors

Recent studies have shown that a variety of xenobiotic chemicals, in addition to disrupting genomic steroid actions, can also interfere with the nongenomic actions of steroids (Nadal et al., 2000; Thomas, 1999, 2000a,b; Thomas and Khan, 2004; Watson et al., 1999). The finding that low concentrations (1 to 10 n*M*, equivalent to 30 ppb) of the xenoestrogens kepone and *o,p′*-DDD interfered with progestogen

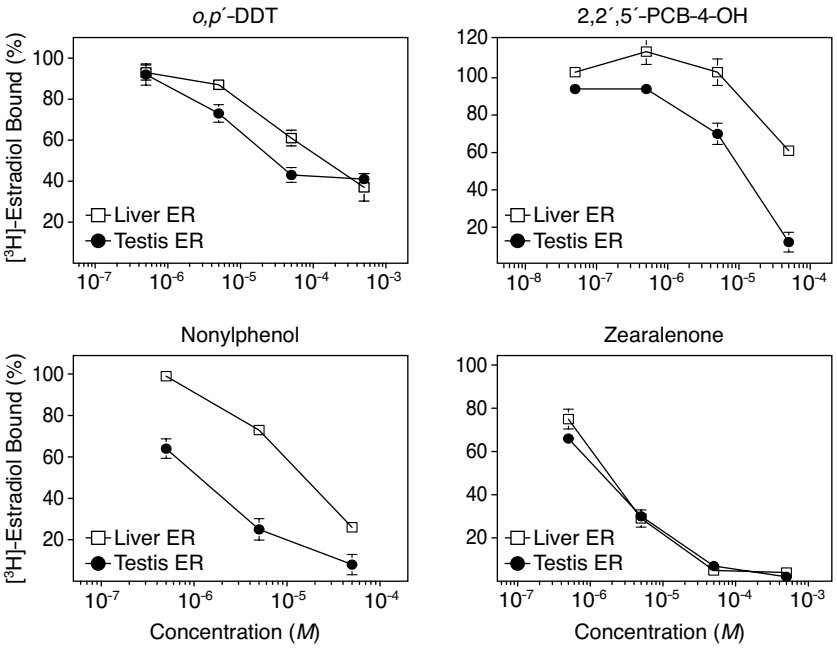

FIGURE 10.6 Comparison of the competition curves of several xenoestrogens for binding to the nuclear estrogen receptor in cytosolic fractions of Atlantic croaker ovarian and hepatic tissues. (From Loomis, A.K. and Thomas, P., *Biol. Reprod.*, 61, 51–60, 1999. With permission.)

induction of meiotic maturation of Atlantic croaker oocytes *in vitro* provided initial evidence for this novel type of endocrine disruption (Ghosh and Thomas, 1995). Subsequently, disruption of oocyte maturation by estrogenic compounds was confirmed in an amphibian species (*Xenopus*) exposed to methoxychlor (Pickford and Morris, 1999). In addition, kepone was shown to partially block the stimulatory actions of progestogens on sperm motility in Atlantic croaker (Thomas et al., 1998). Xenoestrogens such as *o,p′*-DDT and nonylphenol have also been shown to exert rapid estrogenic (agonistic) actions on rat smooth muscle cells and on croaker testicular androgen production (Loomis and Thomas, 2000; Ruehlmann et al.,1998).

Recently, direct evidence has been obtained that estrogenic compounds can disrupt nongenomic 20β-S actions on fish oocytes by a membrane-receptor-mediated mechanism (Das and Thomas, 1999). Competition binding studies have shown that these compounds bind to the mPRs on fish oocytes and sperm and to the membrane estrogen receptor in fish testes and disrupt nongenomic steroid actions in these tissues (Figure 10.6) (Das and Thomas, 1999; Loomis and Thomas, 2000; Thomas et al, 1998). The finding that a wide range of xenoestrogens display similar binding affinities for the mER as they do for the nER in croaker tissues suggests that the hormone actions they mediate may be equally susceptible to interference by these environmental contaminants (see Figure 10.6 and Figure 10.7 for comparison). Xenoestrogen binding to the oocyte mPR can be competitive and reversible; 20β-S receptor binding activity is completely restored by washing the ovarian plasma membranes (Das and Thomas, 1999). Recent evidence suggests that the inhibitory actions of xenoestrogens on 20β-S stimulation of sperm motility is also via completive binding to the receptor. If xenobiotic binding to a hormone receptor is competitive, it should be displaceable with the excess hormone, and the antagonistic actions of the chemical partially reversed (Figure 10.4). This was demonstrated with Atlantic croaker sperm acutely exposed to low concentrations of *o,p′*-DDE (0.1 μ*M*) in the presence of high concentrations (200 n*M*) of 20β-S (Thomas, 2003). Treatment with *o,p′*-DDE alone did not decrease sperm motility below control levels but completely blocked the increase in sperm motility in response to 20-n*M* 20β-S. Exposure of untreated sperm to tenfold higher concentrations of 20β-S (200 n*M*) did not increase motility above that observed with the 20-n*M* 20β-S, but treatment with *o,p′*-DDE fully restored the hormonal response. Partial restoration of sperm motility

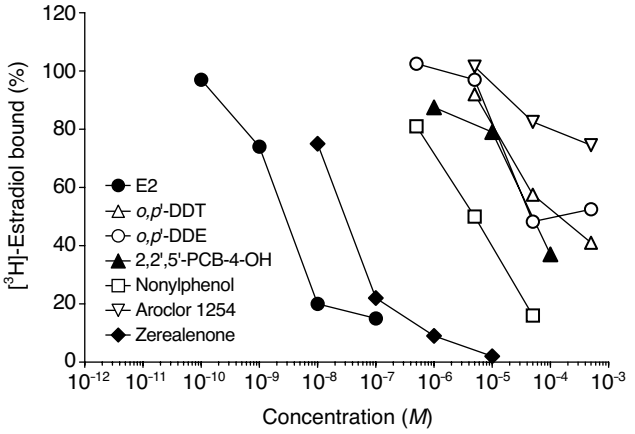

FIGURE 10.7 Competition curves for binding of several xenoestrogens to the membrane estrogen receptor in Atlantic croaker testes. (From Loomis, A.K. and Thomas, P., *Biol. Reprod*., 62, 995–1004, 2000. With permission.)

with excess steroid is also observed with higher concentrations of xenobiotics, as shown in Figure 10.8. Treatment with higher concentrations of 20β-S and another sperm receptor agonist, 11-deoxycorticosterone (conjugated to bovine serum albumen to prevent it from passing through the membrane), partially reverses the inhibitory effects of 2,4,6-PCB-4-OH on progestin-stimulated sperm motility (Figure 10.8). Both *ortho*,*para* DDT derivatives and 2,4,6-PCB-4-OH have previously been shown to compete with 20β-S binding to the croaker sperm mPR (Thomas et al., 1998). Recent evidence suggests that this nongenomic steroid action is especially sensitive to inference by low concentrations (0.01 to 0.01 μ*M*) of a wide range of environmental contaminants (Thomas and Doughty, 2004).

Hypothalamic–Pituitary–Interrenal Axis

The interrenal gland in fishes, which is homologous to the adrenal cortex of mammals, secretes corticosteroid (C-21 steroids) hormones. Cortisol is the principal teleost corticosteroid hormone in fishes, and it functions as both a glucocorticoid and mineralocorticoid hormone, regulating intermediary metabolism and electrolyte balance, respectively. Cortisol is critically important in the regulation of glucose biosynthesis by the liver (gluconeogenesis) and in adaptation to hypersaline environments by regulating chloride cell Na^+/K^+-ATPase activity and ionic efflux by the gills. Cortisol also rapidly decreases the release of prolactin, a critical hormone for adaptation to freshwater, in tilapia, and studies suggest that this cortisol action is nongenomic and is initiated at the cell surface (Borski et al., 2001). Rapid increases in corticosteroid hormones, in concert with increases in catecholamine secretion, occur in response to a wide range of stressors. These hormones regulate a wide variety of adaptive responses that may be critical for survival over the short term, including an increase in the production of metabolic reserves; however, prolonged stressor-induced elevations of cortisol can also impair reproductive and immune functions and negatively influence growth by stimulating protein catabolism.

The synthesis and secretion of corticosteroid hormones by the interrenal glands are controlled by hormones secreted by the hypothalamic–pituitary axis. Corticotropin (adrenocorticotropic hormone [ACTH]), a 39-amino-acid peptide secreted by corticotroph cells in the anterior pituitary, is the primary pituitary hormone regulating corticosteroid secretion. Corticotropin and a variety of other pituitary hormones, such as melanotropin (melanocyte-stimulating hormone [MSH]), are produced from different parts of a precursor protein, proopiomelanocortin. The secretion of ACTH, in turn, is controlled by corticotropin-releasing hormone (CRH), a peptide synthesized in specialized neurosecretory neurons in the nucleus preopticus and nucleus lateralis regions of the hypothalamus and released at the axon terminals in the vicinity of the ACTH-producing cells (corticotrophs) in the anterior pituitary. The

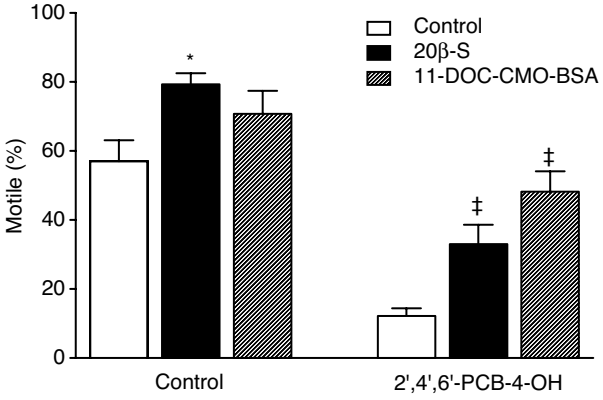

FIGURE 10.8 Stimulatory effects of 5-minute treatment with 20-n*M* 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) or 50-n*M* 11-deoxycorticosterone conjugated to bovine serum albumen (11-DOC-CMO-BSA) on sperm motility in Atlantic croaker *in vitro*. Cotreatment with 50-μ*M* 2′,4′,6′-PCB-OH alone (clear bar on right-hand side of graph) and in the presence these low concentrations of the steroids (results not shown) caused a similar inhibition of sperm motility. A tenfold increase in progestin concentrations (20β-S, 200 n*M*; 11-DOC-CMO-BSA, 500 n*M*; shaded bars on right-hand side of graph) partially reversed the inhibitory effect of the PCB. *Significantly different from no treatment control; ‡significantly different from PCB treatment alone ($n = 6$).

structure of CRH is remarkably conserved among vertebrates, the teleost form differing from the mammalian CRH only at two of the 42-amino-acid residues (Lederis, 1987). In addition, other structurally related peptides, such as urotensin I, which is produced by a caudal neurosecretory organ unique to teleosts (the urophysis), and neurohypophyseal peptides such as argine vasotocin have also been shown to stimulate ACTH secretion (Lederis et al., 1994). Details of the neuroendocrine pathways, neuropeptides, and neurotransmitters involved in activation of the HPI axis in teleosts in response to stressors are currently lacking; however, recent results suggest that serotinergic pathways and CRH mediate the stimulation of ACTH secretion in response to certain stressors (Ando et al., 1999; Winberg et al., 1997), as has been shown in mammals. Activation of the HPI axis is rapid after exposure to many stressors; plasma cortisol levels are often elevated within 5 minutes and reach maximum levels, up to 100 times initial levels within 30 to 60 minutes (Thomas, 1999). Cortisol exerts negative feedback actions on CRH and urotensin I synthesis in the hypothalamus and ACTH secretion from the pituitary (Bernier et al., 1999; Fryer et al., 1984).

Interference of the HPI Axis

Many chemicals, including heavy metals (Brodeur et al., 1998; Donaldson and Dye, 1975; Leblond and Hontela, 1999), polycyclic aromatic hydrocarbons (McFarlane and Benville, 1986; Thomas and Neff, 1985; Thomas et al., 1980; Wilson et al., 1998), organochlorine pesticides (Benguira et al., 2002; Ilan and Yaron, 1980), and nonchlorinated pesticides, have been shown to influence the activity of the HPI axis in teleosts. A wide variety of chemicals and other adverse environmental stimuli elicit a nonspecific stress response in teleosts that is characterized by activation of the sympathetic adrenergic system and HPI axis, resulting in a suite of secondary stress responses, including hyperglycemia, alterations of protein and lipid metabolism, osmotic disturbances, and altered immune function (Mazeaud et al., 1977). Exposure to relatively low concentrations of many chemicals causes an acute elevation in plasma cortisol levels within minutes to a few hours, but the response is usually transient if the treatment is sublethal, and circulating titers often have returned to control levels by 6 hours after the initial exposure (Donaldson and Dye, 1975; MacFarlane and Benville, 1986; Thomas et al., 1980, 1981a). In contrast, cadmium failed to elicit corticosteroid stress responses in Coho salmon and striped mullet (Schreck and Lorz, 1978; Thomas and Neff, 1985). Schreck and Lorz (1978) concluded from their results that only stimuli that cause "fright, discomfort or pain" are capable of inducing a corticosteroid stress response. These acute corticosteroid responses in teleosts to chemicals are similar to those induced by many moderate

physical stressors and have been considered analogous to the initial alarm phase of the general adaptation syndrome in mammals (Mazeaud et al., 1977; Schreck, 1981; Selye, 1950). After this initial response, plasma cortisol often declines to controls levels even though the chemical is still present (Donaldson and Dye, 1975; Thomas and Neff, 1981; Thomas et al., 1981a). This compensation or resistance phase will persist if the chemical stress is moderate (Schreck, 1981; Thomas, 1990); however, if prolonged exposure results in significant bioaccumulation of the chemical and toxicity, cortisol levels increase again and typically remain elevated until homeostasis can no longer be maintained and mortality occurs (Thomas et al., 1981a,b), equivalent to the exhaustion phase described by Selye (1950).

Chemicals also affect the activity of the HPI axis by specific endocrine toxic mechanisms. An early survey of the mammalian literature showed that the adrenal in mammals is the most sensitive endocrine gland to chemical-induced injury (Ribelin, 1984). Chemicals have been shown to impair adrenocortical structure and function via a variety of mechanisms, including covalent binding to proteins, metabolic activation, peroxidative damage, and reversible interactions with steroidogenic enzymes (Colby and Longhurst, 1992). Interrenal function in teleosts also appears to be especially sensitive to chemicals (Bisson and Hontela, 2002), and preliminary evidence suggests that they impair steroidogenesis by similar mechanisms; for example, a decline in cortisol secretion after exposure to the organochlorine pesticide endosulfan is associated with oxidative stress in rainbow trout interrenal steroidogenic cells (Dorval et al., 2003). In this study, the steroidogenic responses to ACTH and the cAMP analog dbcAMP were impaired, suggesting that the site of endosulfan action is downstream from cAMP generation (Dorval et al., 2003). Similar conclusions were drawn from studies involving exposure of the interrenal cells of rainbow trout to the heavy metals cadmium, mercury, and zinc (Leblond and Hontela, 1999). In contrast, tilapia exposed to *o,p′*-DDD showed an impaired response to ACTH but not to cAMP, which suggests that the site of action of *o,p′*-DDD is further upstream, possibly via downregulation or desensitization of the ACTH receptor (Ilan and Yaron, 1980). A recent study in rainbow trout, however, suggests that *o,p′*-DDD impairs corticosteroid production by interfering with interrenal cAMP generation (Lacroix and Hontela, 2003).

No evidence has been obtained to date that xenobiotic chemicals alter the activity of the HPI axis in teleosts by binding to nuclear glucocorticoid receptors. Several alkylphenols and phthalates, the pesticides dieldrin and toxaphene, and the phytoestrogen genestein showed no binding affinity for the rainbow trout nuclear glucocorticoid receptor (Knudsen and Pottinger, 1999).

Hypothalamic–Pituitary–Thyroid Axis

Thyroid hormones have essential functions in the regulation of embryological development, metabolism, and reproduction in vertebrates and also have important roles in metamorphosis and osmoregulation in fishes. The secretory activity of the thyroid in fishes, like that in mammals, is regulated by a pituitary glycoprotein hormone: thyrotropin (TSH). Unlike mammals, however, only minor amounts of the active form of thyroid hormone, triiodothyronine (T_3), are secreted in fishes, the majority being in the form of the prohormone thyroxine (T_4); consequently, the production of the active form of thyroid hormone (T_3) from the prohormone (T_4) in several fish species is largely under peripheral control by the enzyme monodeiodinase (Eales et al., 1999). TSH has a structure similar to that of the gonadotropins, consisting of a shared alpha subunit and a hormone-specific beta subunit. TSH secretion is under neuroendocrine control by a hypothalamic tripeptide, thyrotropin-releasing hormone (TRH), that exerts an inhibitory action on the release of TSH from the pituitary in some teleost species, although in mammals its action is stimulatory (Leatherland, 2000). In addition, evidence suggests that CRH influences the activity of the pituitary–thyroid axis in some fishes and therefore has the potential to be a common mediator of chemical-induced changes in the activities of both the HPI and HPT axes. TSH stimulates the synthesis and release of T_4 from the thyroid gland. An important feature of the hypothalamic–pituitary–thyroid axis is the presence of a negative feedback action of T_4 on TSH secretion, thereby regulating the synthesis of T_4 within a physiological range (Eales et al., 1999). The thyroid gland in most teleosts consists of scattered follicles comprising a single layer of epithelial cells surrounding a fluid-filled cavity containing thyroglobulin, a large colloidal iodinated protein. The epithelial cells (thyrocytes) concentrate iodine

from the blood and transport it to the lumen surface, where it is oxidized by an enzyme (iodoperoxidase or thyroid peroxidase). This enzyme also catalyzes the oxidation of certain tyrosines in the thyroglobulin molecule, resulting in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT) and subsequently the oxidative coupling of these iodinated tyrosines to form the thyroid hormones.

Tetraiodothyronine (T_4) is produced by coupling DIT with DIT and is the major thyroid hormone product in fish, whereas triiodothyronine (T_3), which is made from coupling MIT with DIT, is produced in much smaller amounts. By a process called *pinocytosis*, the thyrocytes, upon stimulation by TSH, incorporate vesicles of thyroglobulin into their cytoplasm, where they fuse with lysosomes, and the thyroglobulin is enzymatically degraded to yield the thyroid hormones. The thyroid hormones are subsequently released into the circulation and are transported in the blood in most teleost species bound to carrier proteins (thyroid-binding globulins). The carrier proteins typically have a higher affinity for T_4 than T_3 and serve as a buffer and reservoir for these hormones. Thyroxine is converted in several tissues including the kidney, brain, and liver to the physiologically active hormone T_3 by removal of an iodide from the outer tyrosine ring by the enzyme 5′-monodeiodinase. A related enzyme, 5-monodeiodinase, which is present in certain tissues, catalyzes the removal of iodide from the inner tyrosine ring to produce reverse T_3, which is physiologically inactive. The two monodeiodinases further deiodinate T_3 and reverse T_3 to form thyronine, a major route of degradation of thyroid hormones prior to excretion; thus 5′- and 5- monodeiodinases have critical roles in T_3 homeostasis, as they regulate both the synthesis and degradation of this physiologically active form of the hormone. T_3 is lipid soluble (lipophilic) and readily diffuses into target cells, where it binds to the thyroid receptor (TR), which belongs to the same superfamily of ligand-activated transcription factors as nuclear steroid receptors. Similarly, T_3 binding to the TR results in dimerization, but, unlike the steroid receptors, thyroid receptors form heterodimers with retinoid X receptors (RXRs) to form the activated hormone–receptor complex. The TR component of this complex binds to thyroid hormone response elements on genes, resulting in alterations in their rates of transcription. There is also evidence for nongenomic actions of thyroid hormones initiated at the cell surface in several mammalian models (Norman, 2004).

Interference of the HPT Axis

A variety of environmental chemicals, including PCBs, insecticides, water-soluble fractions of oil, aluminum, lead, and cadmium, have been shown to alter the thyroid system in fishes by causing decreases in the circulating levels of thyroid hormones and hepatic 5′-monodeiodinase activity (Chaurasia et al., 1996; Fok et al., 1990; Folmar et al., 1982; Hontela et al., 1996; Leatherland and Sonstegard, 1978; Sinha et al., 1992), although the sites and mechanisms of chemical interference with thyroid function remain poorly understood. Declines in monodeiodinase activity have been reported in fishes after *in vivo* exposure to metals and insecticides (Chaurasia et al., 1996; Fok et al., 1990), but it is unclear whether these chemicals are acting directly on the enzyme or indirectly by altering other endocrine systems that influence monodeiodinase activity (Eales et al., 1999). Deiodinases contain selenium bound to cysteines and therefore are potentially susceptible to interference by heavy metals that displace selenium from these sites in proteins such as cadmium, mercury, and copper. Deiodination has been proposed as a valuable biomarker of interference of the thyroidal system in fish (Eales et al., 1999). Other potential sites of xenobiotic interference with thyroid function include interference with neurotransmitter control of neuroendocrine function (Thomas and Khan, 2004) and binding to the thyroid hormone receptor or plasma transport protein, although direct evidence is lacking (see reviews by Leatherland, 2000; Eales et al., 1999; Brown et al., 2004).

Summary

The physiological functions of the gonads and interrenal and thyroid glands in fishes, like those of tetrapods, are under complex neuroendocrine control by hormones synthesized by the hypothalamus and pituitary. The hormones secreted by the gonads and interrenal and thyroid glands in turn exert negative feedback effects on neuroendocrine function; however, the thyroid hormone function in fishes is largely

controlled peripherally by monodeiodinases, the enzymes that convert T_4 to the active form of the hormone (T_3) or to inactive metabolites. Sex steroid hormones synthesized by the gonads control critical functions in this tissue during gametogenesis (paracrine control) in addition to exerting their actions at other target tissues. Chemicals could potentially disrupt these complex endocrine systems at multiple sites along their axes and via a variety of mechanisms. Chemicals can disrupt endocrine function by altering the circulating levels of hormones, by decreasing hormone secretion, by increasing hormone metabolism, or by interfering with hormone action. Some examples in teleosts of each of these mechanisms of endocrine toxicity have been briefly reviewed. Novel mechanisms of toxicity such as interference with nongenomic steroid actions and neuroendocrine dysfunction resulting from impaired hypothalamic neurotransmitter function were first identified in fishes. Information on the mechanisms of endocrine toxicity can often be used to develop biomarkers of endocrine impairment specific for particular types of chemicals. These biomarkers have been used in environmental assessments of the health of fish populations in impacted environments; for example, the presence of the estrogen inducible proteins such as vitellogenin in male and juvenile fish has proven useful for detecting the presence of xenoestrogens in environmental samples.

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11

The Immune System of Fish: A Target Organ of Toxicity

Erik Carlson and Judith T. Zelikoff

CONTENTS

Fish Immunology

Over the past few decades, our understanding of fish immunology has greatly progressed due to studies utilizing molecular techniques, cell culture, and monoclonal antibodies. Most immunological research involving fish species has been concerned with either their phylogenetic or economic importance; thus, such groups as agnathans, elasmobranchs, and chondrosteans have been studied in attempts to elucidate the evolution of the vertebrate immune system. Also, species such as salmonids (*Oncorhynchus* spp. and *Salmo* spp.), carp (*Cyprinus* spp.), and catfish (*Ictalurus* spp.) have received interest mainly due to their use in aquacultural settings. This section primarily focuses upon teleost fish and briefly reviews current research in the area of fish immunology. The reader is directed toward reviews by Iwama and Nakanishi (1996), Bernstein et al. (1998), and Pastoret et al. (1998) for additional information in this rapidly expanding field. Following an introduction to the major lymphoid cells/tissues of teleosts, nonspecific and specific components of the teleost immune system are presented in this chapter. Both cellular (i.e.,

macrophages, granulocytes, and natural-killer-like cells) and noncellular (i.e., lysozyme, complement, and acute-phase proteins) aspects of nonspecific immunity, as well as specific (adaptive) immune responses that rely upon acquired immunity (i.e., cell- and humoral-mediated immunity), are discussed.

Immune Cells

Many morphological and functional similarities exist between mammalian and teleostean leukocytes (Rowley et al., 1988). Lymphocytes, macrophages (MØs)/monocytes, granulocytes (neutrophilic, eosinophilic, and basophilic), and nonspecific cytotoxic cells (NCCs) have all been described in a variety of teleost models; however, species-specific differences in occurrence, morphology, and function are common. These differences make generalizations about fish leukocytes difficult. Cells involved in the nonspecific cellular response include MØs, granulocytes, and NCCs. All of these cell types are vitally important for inflammation and probably represent the most primitive group of immune-effector cells. Although excellent descriptions of MØs (Secombes, 1996; Secombes and Fletcher, 1992), granulocytes (Ainsworth, 1992; Secombes, 1994; Suzuki and Iida, 1992), and NCCs (Evans and Jaso-Friedmann, 1992; Secombes, 1996) can be found elsewhere, a brief review of cell function is provided here (see discussion on nonspecific immune defenses). It appears that jawed vertebrates (gnathostomes) are the most phylogenetically primitive organisms to possess lymphocytes. Both B- and T-lymphocytes have been described in teleosts due primarily to functional and structural similarities shared by their mammalian counterparts. The presence of antibody in bony fish led early investigators to believe that fish possessed antibody-producing B-lymphocytes. The expression of surface immunoglobulin (sIg) on fish lymphocytes has since been described in a number of species, including channel catfish (*Ictalurus punctatus*) (Lobb and Clem, 1982), carp (*Cyprinus carpio*) (Koumans-van Diepen et al., 1994), and sea bass (*Dicentrarchus labrax*) (Romestand et al., 1995). Teleost T-lymphocytes are generally recognized as surface immunoglobulin-negative lymphocytic cells, and T-lymphocyte-specific monoclonal antibodies have been produced for some species (Partula, 1999; Scapigliati et al., 1999). The structure and known functions of fish lymphocytes are described in detail later in this section.

Lymphoid Organs and Tissues

The lymphoid tissues and organs of teleost fish have previously been reviewed (Press and Evensen, 1999; Zapata et al., 1996). Unlike mammals, fish lack hematopoietic bone marrow and identifiable lymph nodes. The primary site of hematopoiesis in teleosts is the pronephros or anterior portion of the kidney (commonly referred to as the head or cranial kidney). Teleosts also possess a thymus and spleen (although splenic germinal centers are absent) along with mucosal lymphoid tissues. The morphology of fish lymphoid organs is highly species specific, although some generalizations about structure and function can be made.

Thymus

In most teleosts, the thymus is a paired organ located dorsolaterally above the opercular cavities (Chilmonczyk, 1992). The thymus has been found anywhere from just beneath the opercular epithelium, as in rainbow trout (*Oncorhynchus mykiss*) (Chilmonczyk, 1985) to a more internalized location as described in angler fish (Fange and Pulsford, 1985). Histologically, the thymus is poorly differentiated into cortical and medullary regions, with as many as six different zones of varying cell densities (Zapata et al., 1996). Figure 11.1 demonstrates the thymus from Japanese medaka (*Oryzias latipes*).

As in higher vertebrates, the fish thymus is believed to be the first lymphoid tissue to become populated by lymphocytes during development. The ontogenesis of the thymus gland has been described for many fish species, including rainbow trout (*Oncorhynchus mykiss*) (Chilmonczyk, 1985; Grace and Manning, 1980), carp (*Cyprinus carpio*) (Romano et al., 1999a; van Loon et al., 1982), zebrafish (*Danio rerio*) (Willett et al., 1997, 1999), sea bass (*Dicentrarchus labrax*) (Abelli et al., 1996), catfish (*Ictalurus punctatus*) (Petrie-Hanson and Ainsworth, 2001), and tilapia (*Oreochromis niloticus*) (Fischelson, 1995). Involution of the teleost thymus has also been described following exposure to various environmental

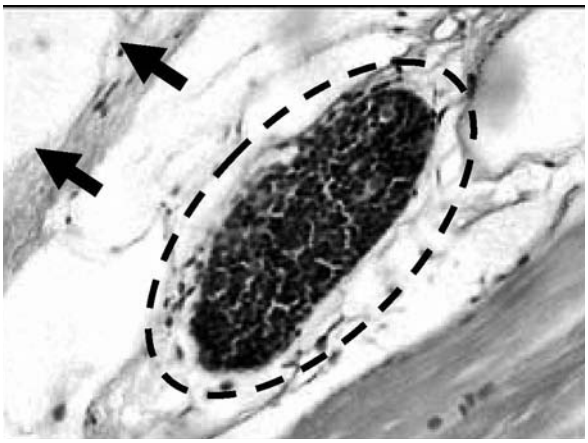

FIGURE 11.1 (See color insert following page 492.) Teleost thymus as represented by the Japanese medaka (*Oryzias latipes*). Note the close proximity of the organ (dashed circle) to the opercular epithelium (arrows). Hematoxylin and eosin-stained light micrograph (100× magnification).

stimuli (Alvarez et al., 1998) and during the aging process (Cooper et al., 1983; Ghoneum and Egami, 1982; Ellsaesser et al., 1988; Fishelson, 1995). Besides being populated by both sIg^- and sIg^+ lymphocytes (Scapigliati et al., 1999), the thymus has been observed to possess epithelial cells of varying morphologies (Castillo et al., 1990; Romano et al., 1999a,b); MØs and monocytes (Castillo et al., 1990); myeloid cells (Zapata et al., 1996); Hassell's body-like structures (Romano et al., 1999a,b); nurse cells (Flano et al., 1996; Romano et al., 1999a,b); and neuroendocrine cells (Ottaviani et al., 1995, 1997). Although the function of many of these thymic cell types remains to be determined, there is ample evidence that the teleost thymus serves the same role in T-lymphocyte production in fish as in mammals (Chilmonczyk, 1992). Such evidence includes: (1) migration of radiolabeled lymphocytes through the thymus (Tatner, 1985), (2) Con A-induced proliferation of thymic lymphocytes (Ellsaesser et al., 1988), (3) apoptosis of thymocytes (Abelli et al., 1998), and (4) *rag* gene expression in the thymus (Willett et al., 1997).

Anterior Kidney

The kidney of teleost fish not only contains the excretory tissues also found in mammals (i.e., nephrons) but also houses the medullary and cortical adrenal homologs (i.e., stanniocalcin-secreting organs, or corpuscles of Stannius), and hematopoietic tissue (Figure 11.2). The fish kidney is often situated along the dorsal wall of the body cavity and can sometimes be divided into anterior (head or cranial) and

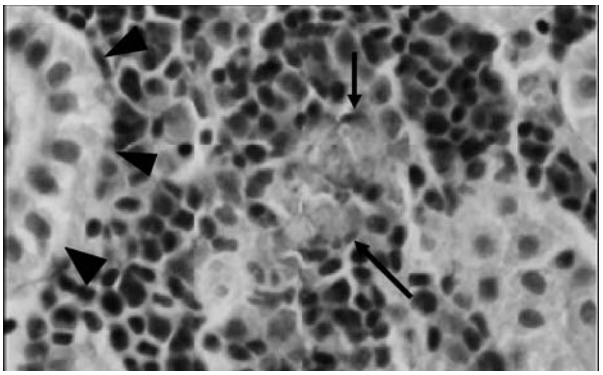

FIGURE 11.2 (See color insert following page 492.) Teleost anterior kidney as represented by the Japanese medaka (*Oryzias latipes*). Hematoxylin and eosin-stained light micrograph (100×) demonstrates kidney hematopoietic tissue interspersed between renal tubules (arrowhead). Note the presence of small macrophage aggregates (arrow).

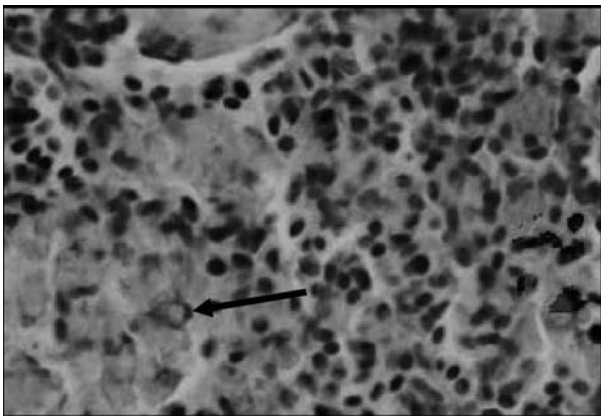

FIGURE 11.3 (See color insert following page 492.) Teleost spleen as represented by the Japanese medaka (*Oryzias latipes*). Hematoxylin and eosin-stained light micrograph (100×) demonstrates hematopoetic splenocytes. Note the macrophage aggregates (arrow).

posterior (trunk or caudal) sections. The anterior kidney is the primary hematopoietic organ in adult teleosts (Press and Evensen, 1999). It appears from studies in cod (*Gadus morhua*) (Schroder et al., 1998), sea bass (*Dicentrarchus labrax*) (Breuil et al., 1997), rainbow trout (*Oncorhynchus mykiss*) (Castillo et al., 1993), and carp (*Cyprinus carpio*) (Koumans-van Diepen et al., 1994; Romano et al., 1997a,b; Secombes et al., 1983) that the anterior kidney is the first lymphoid organ to possess sIg^+ cells during ontogenesis. The aforementioned studies, along with those investigations examining the immune function of anterior kidney cells (Kaattari and Irwin, 1985) suggest that this organ also serves as a secondary lymphoid organ.

Spleen

As in mammals, the spleen is believed to be a secondary lymphoid organ in bony fish (Figure 11.3). In most teleosts, the spleen is an encapsulated organ with abundant red pulp and poorly developed white pulp (Press and Evensen, 1999; Zapata et al., 1996). Unlike the thymus and anterior kidney, the teleost spleen seems to become organized late in the developmental process (Zapata et al., 1996). Although fish lack germinal centers, many immune cells can be found in both splenic red and white pulps (Press et al., 1994; Quesada et al., 1990; Zapata, 1982). The white pulp of teleosts contains poorly developed ellipsoids and numerous MØ aggregations (Press and Evensen, 1999). These so-called MØ aggregates (MAs) or melanomacrophage centers have been observed in a variety of fish species; the occurrence and structure of MAs are species dependent (Aguis, 1980; Wolke, 1992). Further details as to the structure and function of MAs are provided later in this chapter.

Mucosa

Teleosts possess highly diffuse and unorganized lymphoid accumulations consisting of lymphocytes, granulocytes, MØs, and plasma cells (i.e., antibody-forming cells [AFCs]) throughout the gut, gills, and skin (Abelli et al., 1999; Hart et al., 1988; Kaattari and Piganelli, 1996; Zapata et al., 1996). These accumulations occur mainly beneath the epithelium of the gut and in the lamina propria (Hart et al., 1988). Immunohistochemical localization of sIg^- lymphocytes has been observed very early in development (Picchietti et al., 1997). In addition, antibodies have been produced that are specific for carp (*Cyprinus carpio*) mucosal T-lymphocytes and not sIg^- lymphocyte populations found elsewhere (Rombout et al., 1998). This latter finding is interesting, as it describes a distinct T-lymphocyte subpopulation distinguishable from sIg^- lymphocytes that are present in other lymphoid organs. Macrophages and sIg^+ lymphocytes have also been observed in the mucosal lymphoid tissues of carp (Rombout et al., 1993).

Numerous studies have described antigen uptake in the teleost gut (Robohm and Koch, 1995; Rombout et al., 1985), skin, and gills (Moore et al, 1998; Ototake et al., 1996; Tatner et al., 1984; Zapata et al., 1986). Oral and bath/immersion vaccinations have been observed to yield a differential response in the specificity of mucus and serum antibodies without any observable isotypic difference between the secretory and serum antibody molecules (Kaattari and Piganelli, 1996). Investigators have also observed a phenomenon similar to mammalian oral tolerance in rainbow trout (*Oncorhynchus mykiss*) following oral antigen exposure (Jones et al., 1999).

Nonspecific Immunity

Nonspecific mechanisms of an immune response in teleosts include both soluble and cellular components. Soluble constituents are mainly evolutionarily conserved molecules that have broad cross-reactivity, recognizing molecular motifs shared by a wide variety of microorganisms. Nonspecific soluble molecules described in this chapter include complement, acute-phase proteins, and lysozyme. Although cytokines (i.e., interferons, interleukins, tumor necrosis factors, and transforming growth factors) are involved in the regulation of both innate and adaptive immune responses, they are included in this section for simplicity. Cellular components of the nonspecific immune response involved in maintaining internal homeostasis by removing dead or unwanted cells include MØs, granulocytes, and NCCs.

Soluble Components

Complement

The complement system of fish has been reviewed (Sakai, 1992; Sunyer and Lambris, 1998; Yano, 1996). Virucidal, bactericidal, parasiticidal, opsonic, and chemotactic activities of complement components have all been described in teleost species (Yano, 1996). Many teleost fish have been found to possess classical, alternative, and lytic complement components similar to those of mammals. Proteins involved in the lectin-activated pathway have also been described (Endo et al., 1998; Nagai et al., 2000; Nonaka and Smith, 2000). Interestingly, the alternative complement pathway of several fish species are much more pronounced than those described for mammals, suggesting a greater importance for this pathway in fish immune defense (Sunyer et al., 1996; Yano, 1996). Teleosts have been found to possess a more diverse array of complement components than higher vertebrates (Sunyer et al., 1998); for example, multiple C3 forms have been described for rainbow trout (*Oncorhynchus mykiss*) (Sunyer et al., 1996), sea bream (*Sparus aurata*) (Sunyer et al., 1997), and carp (*Cyprinus carpio*) (Nakao et al., 2000), and there is evidence that these isoforms are the products of separate genes. Additionally, due to the presence of a molecule that shares characteristics of both C2 and factor B in bony fish, the emergence of separate molecules appears to have occurred following the divergence of teleost species from the tetrapod line (Kuroda et al., 1996; Seeger et al., 1996; Sunyer et al., 1998).

Interferon

Evidence for the presence of interferon (IFN) in teleosts has been reviewed (Yano, 1996). Teleost IFN activity was originally described in cell culture (Gravell and Malsberger, 1965) and by studies in rainbow trout (*Oncorhynchus mykiss*), demonstrating the induction of a protein with antiviral and MØ activating factor properties (Graham and Secombes, 1990). Recently, sequences of IFN-like genes have been identified in zebrafish (*Danio rerio*) (Altmann et al., 2003) and catfish (*Ictalurus punctatus*) (Long et al., 2004). Induction of antiviral fish Mx proteins by viral infection and poly I:C incubation has also been demonstrated (Lee et al., 2000; Nygaard et al., 2000; Trobridge and Leong, 1995; Trobridge et al., 1997).

Interleukins

Interleukins (ILs) are small molecules involved in the regulation of both innate and adaptive immunity in mammals (Kaiser et al., 2004). Recently, considerable effort has been directed toward the elucidation of interleukin genes in fish species (Secombes and Cunningham, 2004; Secombes et al., 1996, 2001). Mammalian IL-2 is a potent growth factor. Although media from activated fish leukocyte cultures have

been found to possess IL-2-like activity (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984), an attempt to sequence the IL-2 gene of Japanese flatfish (*Paralichthys olivaceus*) resulted in a product with little or no homology to mammalian IL-2 (Tamai et al., 1992, 1993a,b). The proinflammatory interleukin IL-1β has been cloned and characterized in several fish species (Bird et al., 2002), and recombinant rainbow trout IL-$1\beta_1$ activates phagocyte migration and phagocytosis (Secombes et al., 2001). In addition, an ortholog to IL-18 (a related IL-1 family member) has been identified in the pufferfish (*Fugu rubripes*) genome (Huising et al., 2004). A cDNA sequence similar to members of another proinflammatory interleukin family, IL-6, has recently been identified in carp (*Cyprinus carpio*) (Fujiki et al., 2003). Further evidence for an IL-6-like molecule in fish is provided by the observation that antisera directed toward mammalian IL-6 cross-reacts with sera of virally infected carp and trout (Ahne, 1993, 1994). Although correctly classified as a CXC chemokine, IL-8 orthologs have been identified in lamprey (*Lampreta fluviatis*) (Najakshin et al., 1999), Japanese flounder (*Paralichthys olivaceus*) (Lee et al., 2001), and rainbow trout (*Oncorhynchus mykiss*) (Laing et al., 2002).

Tumor Necrosis Factor

Tumor necrosis factor (TNF) family members are involved in immune regulation, apoptosis, and cell proliferation (Goetz et al., 2004). Tumor necrosis factor sequences, similar to those of mammalian TNFα, have been described in several fish species (Bobe and Goetz, 2001; Garcia-Castillo et al., 2002; Hirono et al., 2000; Laing et al., 2001; Saeij et al., 2003; Zou et al., 2003). Catfish TNF was expressed in both lymphoid (spleen, head kidney, and thymus) and non-lymphoid (liver and gill) organs, as well as in peripheral blood leukocytes (Zou et al., 2003). Furthermore, TNF mRNA was induced upon activation of immune cells from flounder, catfish, and rainbow trout (Hirono et al., 2000; Laing et al., 2001; Zou et al., 2003).

Transforming Growth Factor

Members of the transforming growth factor (TGF) superfamily are involved in the regulation of numerous mammalian cellular responses including inflammation (Magor and Magor, 2001). Sequences homologous to mammalian TGF-β have been identified in several fish species (Daniels and Secombes, 1999; Harms et al., 2000; Laing et al., 1999; Magor and Magor, 2001; Zhan and Jimmy, 2000). In addition, mammalian TGF-β_1 was found to modulate rainbow trout MØ respiratory burst activity (Jang et al., 1994).

Lysozyme

Lysozymes, low-molecular-weight antimicrobial factors that destroy Gram-positive bacterial cell walls, can also act as opsonins to enhance phagocytosis. Fish lysozyme has been found to be present in head kidney, skin, digestive tract, and gills, as well as intracellularly within neutrophils, monocytes, and eosinophilic granule cells (Yano, 1996). One unique feature of fish lysozyme is that it appears to be effective against both Gram-positive and -negative bacteria, although lysozyme-mediated Gram-negative bacterial lysis requires the presence of complement (Grinde, 1989).

Miscellaneous Acute-Phase Proteins and Soluble Factors

Evidence for the existence of various other soluble compounds involved in teleost innate immunity—C-reactive proteins (CRPs), transferrins, lectins, α_2-macroglobulin, and antimicrobial peptides—has been reported (Alexander and Ingram, 1992; Bayne and Gerwick, 2001; Magor and Magor, 2001; Yano, 1996). Serum CRP has been determined in various fish species (Bayne and Gerwick, 2001), including rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), and plaice (*Pleuronectes platessa*). In addition, evidence exists for other compounds (present in serum and in mucus of the digestive tract, gills, and skin) that may be involved in the acute-phase response. These include transferrin (Bayne and Gerwick, 2001; Yano, 1996), serum amyloid A and P (Jensen et al., 1997; Jorgensen et al., 2000; Lund and Olafsen, 1999), pre-cerebellin-like protein (Gerwick et al., 2000), pleurocidin (Cole et al., 1997; Douglas et al., 2001), α_2-macroglobulin (Mutsuro et al., 2000), cathepsins (Aranishi and Mano, 2000), histone-like proteins (Noga et al., 2001, 2002; Robinette and Noga, 2001), and lectins (Fujiki et al., 2001; Inagawa et al., 2001; Nakamura et al., 2001; Yano, 1996).

Cellular Response

Monocytes/Macrophages

Macrophages, terminally differentiated mononuclear cells derived from circulating blood monocytes, have been well studied in mammals and a variety of fish species (Enane et al., 1993; Secombes, 1996; Secombes and Fletcher, 1992). As in mammals, several distinct MØ subpopulations appear to exist in fish (Neumann et al., 2000). Although investigations regarding the distribution of monocytes/MØs in adult fish and during development have been hampered due to a lack of readily available MØ-specific markers, studies by Romano et al. (1997) demonstrated MØs in carp (*Cyprinus carpio*) head kidney as early as 2 days after fertilization; by 2 weeks of age, MØs were observed in almost all lymphoid tissues. Phagocytosis, a critical function of monocytes/MØs and likely one of the most ancient immune mechanisms, has been the subject of intense investigation in various fish species (Secombes and Fletcher, 1992). Following uptake, engulfed pathogens are destroyed intracellularly via the production of reactive oxygen/nitrogen species and oxygen-independent mechanisms (Neumann et al., 2001). Several immunoregulatory molecules such as MØ-activating factor (MAF), TNF-α, TGF-β, β-glucans, growth hormone, and neurotransmitters have been implicated in the modulation of fish MØ phagocytosis (Secombes, 1994, 1996).

Of interest is the occurrence in a variety of fish species of focal aggregations of pigmented MØ populations. Macrophage aggregates are found in a variety of ectothermic vertebrates and are commonly occurring among piscine species (Agius, 1980; Wolke, 1992). These aggregates, appearing anywhere from yellow to black in color, have been found to contain hemosiderin, lipofuscin (ceroid), or melanin pigments. The gross anatomical and histological morphologies of MAs are highly species dependent. In some species such as smallmouth bass (*Micropterus dolomieu*), they are yellow in color and occur as highly organized, nodular structures. In species such as rainbow trout (*Oncorhynchus mykiss*), these aggregates are small and poorly organized and appear darkly pigmented (Wolke, 1992). Tissue distribution of MAs is species dependent, but they are commonly observed in both lymphoid (i.e., spleen, kidney, and thymus) and non-lymphoid (i.e., liver, gonads, and heart) tissues. Size, pigmentation, and number of MAs appear to be impacted by environmental change, both natural and anthropogenic; however, high interindividual variation in such parameters often precludes the use of MAs as a biomarker (Wolke, 1992). The immunological relevance of MA in fish is highly controversial. Some believe that they serve as primordial germinal centers that function in antigen trapping and presentation (Wolke, 1992). Others speculate that they serve as no more than "tissue dumpsters" that collect and harbor cellular byproducts and debris or as "recycling bins" involved in the destruction of red blood cells (Zapata et al., 1996).

Granulocytes

Attempts to classify the granulocytic (heterophilic) cell types of teleosts according to mammalian nomenclature have been hampered by tremendous morphological variations in granulocyte subpopulations and the common occurrence of immature (blast) cells in circulation (Ainsworth, 1992; Rowley et al., 1988). Although some fish species (i.e., rainbow trout) do possess neutrophilic granulocytes with polymorphonuclear morphology akin to mammalian polymorphonuclear neutrophils (PMNs), the granulocytes of most fish species lack such a clear nuclear adaptation. Despite difficulties in classification and species-specific variations, neutrophilic, eosinophilic, and basophilic granulocytes have been described for many fish species (Ainsworth, 1992; Reite, 1997,1998; Suzuki and Iida, 1992).

Typical functions of mammalian PMN during inflammation such as phagocytosis, respiratory burst activity, and chemotaxis, have also been observed for fish granulocytes (Ainsworth, 1992; Rowley et al., 1988). As in mammals, the PMN of many teleost species are the first immune cell type to emigrate to the site of inflammation. In some cases, this has been followed by a subsequent decrease in neutrophil number, indicating that certain hematopoetic tissues may serve in neutrophil storage (Suzuki and Iida, 1992).

Like for their mammalian counterparts, evidence exists in fish that eosinophilic granulocytes can also be phagocytic and destroy ingested pathogens (Bodammer, 1986; Cone and Wilkes, 1985; Huizinga, 1980; Sharp et al., 1989). In addition, eosinophilic granulocytes of tilapia (*Oreochromis niloticus*) (Matsuyama and Iida, 1999), rainbow trout (*Oncorhynchus mykiss*) (Ellis, 1985; Powell et al., 1991,

1993), and Atlantic salmon (*Salmo salar*) (Reite and Evansen, 1994) have been found to degranulate upon inoculation with certain noxious or infectious agents. Degranulation in tilapia was correlated with increased neutrophil migration to the injection site, suggesting that fish eosinophils may be analogous to mammalian mast cells (Matsuyama and Iida, 1999).

Nonspecific Cytotoxic Cells

Many teleost species possess immune cells involved in spontaneous, nonspecific cytotoxicity against transformed mammalian cells, parasites, and viral/bacterially infected cells (Evans and Jaso-Friedmann, 1992; Secombes, 1996; Shen et al., 2002). A cell surface marker (isolated from catfish) found to be important in teleost NCC activity appears to be conserved between mammalian species and fish (Jaso-Friedmann et al., 1997, 2001). Nonspecific cytotoxic cells are usually found in the head kidney, spleen, and blood (Evans and Jaso-Friedmann, 1992); circulating NCCs are morphologically and functionally distinct from those of the pronephros and spleen (Hogan et al., 1996; Shen et al., 2002).

Adaptive Immune Defense

Adaptive (acquired) immunity in mammalian species involves: (1) recognition of foreign, non-self entities by antigen-specific lymphocytes, (2) proliferation and differentiation of antigen-specific lymphocyte clones, and (3) memory and enhanced response upon subsequent exposure to antigen (due to greater numbers of antigen-specific lymphocytes). The nature of the adaptive immune response depends upon the activation of either T-lymphocytes (i.e., cell-mediated immunity) or B-lymphocytes (i.e., humoral immunity). Teleost fish possess the genetic machinery necessary to mount both humoral- and cell-mediated immune responses (Kaattari and Piganelli, 1996; Manning and Nakanishi, 1996). This includes genes encoding recombination activating genes (RAGs) (Willett et al., 1997), major histocompatibility complex (MHC) class I and II (Stet et al., 1998), T-cell receptors (TCRs) (Haire et al., 2000; Partula et al., 1999; Wermenstam and Pilstrom, 2001; Wilson et al., 1998; Zhou et al., 1997), immunoglobulin heavy and light chains (Pilstrom and Bengten, 1996), cytokines (Secombes et al., 1999), and various immune coreceptors (Hansen and Strassburger, 2000; Yoder et al., 2001). In addition, the functional equivalents of antigen presenting cells (APCs) are present in fish (Miller et al., 1994; Vallejo et al., 1991, 1992). Fish species have also been shown to exhibit antigen-specific antibody production (Kaattari and Piganelli, 1996), cytotoxic T-lymphocyte (CTL) activity (Stuge et al., 2000; Verlhac et al., 1990; Yoshida et al., 1995), mixed-lymphocyte responses (Miller et al., 1986), delayed hypersensitivity (Manning and Nakanishi, 1996), graft-vs.-host reactions (Nakanishi, 1994), and xenograft/allograft rejection (Manning and Nakanishi, 1996).

Humoral Immunity

The development of teleost immune cell lines has greatly advanced our understanding of the fish humoral response (Miller et al., 1998). B-lymphocytes of fish express both B-cell receptors (BCRs) and secreted forms of IgM. Cross-linking of BCRs results in protein tyrosine phosphorylation, calcium ion influx, and B-lymphocyte proliferation (Van Ginkel et al., 1994). Elicitation of anti-hapten responses to T-lymphocyte-independent antigen in catfish has been shown to require APCs and B-lymphocytes but not T-lymphocytes (Miller et al., 1985). The requirement for APCs (i.e., MØs) in this response is due to the need for IL-1 release (Ortega, 1993). In addition, responses to T-lymphocyte-dependent antigens appear to require the presence of T- and B-lymphocytes along with APCs. The T-lymphocyte-dependent response requires that antigen be processed by APCs prior to elicitation of the response. The role that MHC molecules play in humoral immunity in fish has yet to be clearly defined (Vallejo et al., 1990). Various effector mechanisms have been described for teleost antibody, including neutralization, opsonization, complement fixation, precipitation, and agglutination (Kaattari and Piganelli, 1996). In addition, protective antibodies against bacterial and viral pathogens are produced following immunization. Although teleosts do not appear to possess a secondary humoral response as dramatic as that seen in mammals, secondary exposure of fish to sub-immunogenic doses of an antigen elicits a significantly greater response compared to that observed following the initial exposure (Arkoosh and Kaattari, 1991).

Until recently, teleost antibody was thought to be of only the IgM isotype that exists as a tetramer comprised of the classical two heavy-chain and two light-chain (H_2L_2) subunit structure (Kaattari and Piganelli, 1996); however, recent investigations in two teleost species have described a second isotype similar to mammalian IgD (Miller et al., 1998). Although it appears that fish possess limited isotypic diversity, several studies have indicated some heterogeneity of IgM heavy chains (Lobb and Olson, 1988) and isotypic variants of light chains (Lobb et al., 1984; Sanchez and Dominguez, 1991; Whittington, 1993). Several investigators have also shown that subpopulations of teleost IgM exist that differ in covalent structure (Lobb, 1986; Lobb and Clem, 1981, 1983; Warr, 1983; Whittington, 1993). The tetrameric molecules appear to contain various configurations in which the presence of both inter-heavy and inter-subunit disulfide bonds varies. This produces the appearance of an array of protein bands from 90 to 750 kDa under nonreducing, denaturing SDS-PAGE electrophoresis (Kaattari and Piganelli, 1996). The gene arrangement of the teleost heavy chain is similar to that described for mammals, with variable (V_H), diversifying (D), and joining (J_H) regions upstream of the constant segments (Miller et al., 1998). The light-chain gene organization is similar to that of elasmobranchs in that V_L, J_L, and constant (C_L) segments appear in multiple clusters (Miller et al., 1998). In addition, teleost B-cells demonstrate Ig heavy-chain germline rearrangements and allelic exclusion (Miller et al., 1994).

Cell-Mediated Immunity

Until just a few years ago, characterization of specific cell-mediated cytotoxicity in teleost species had been hampered due to a lack of defined cellular and molecular markers (Stuge et al., 2000). Early studies of cell-mediated immunity in fish demonstrated that acute (less than 14-day) allograft rejections of skin and scales occur in many teleost species (Manning and Nakanishi, 1996). The cell-mediated phenomenon of graft-vs.-host reaction has also been described in studies that employed triploid and tetraploid ginbuna–goldfish (*Carassius auratus*) hybrids (Nakanishi and Ototake, 1999). Finally, delayed hypersensitivity responses (DHRs) have been recorded in response to bacterial and parasitic antigens in various fish species (Manning and Nakanishi, 1996). Recently, allospecific cytotoxic responses of fish leukocytes have been demonstrated *in vitro* (Fisher et al., 1998). In addition, intricate studies utilizing TNP-modified allogeneic and autologous cells have indicated that genetically restricted target cell recognition is involved in the specific CTL response of fish following both *in vitro* and *in vivo* priming (Hasegawa et al., 1999; Verlhac et al., 1990). The discovery of TCR-α and -β chain sequences in fish (Zhou et al., 1997) has allowed for the demonstration of at least four subpopulations of cells involved in the CTL response of channel catfish (*Ictalurus punctatus*) (Stuge et al., 2000). One population was identified as TCR$\alpha\beta^+$ allospecific cytotoxic cells (i.e., equivalent to mammalian CTL); other groups included TCR$\alpha\beta^+$ nonspecific cytotoxic cells, TCR$\alpha\beta^-$ allospecific cytotoxic cells, and TCR$\alpha\beta^-$ nonspecific cytotoxic cells. In addition, a TCR$\alpha\beta^+$ cell subpopulation that proliferated in response to allogeneic stimulation, but possessed no cytotoxicity toward target cells, was also observed. It is thought that the TCR$\alpha\beta^+$ subpopulation may represent a T-helper lymphocyte population (Stuge et al., 2000).

Immunomodulation by Chemicals

Immunotoxicology, a specific subspecialty within the field of toxicology, has gained increasing interest in the last decade due primarily to the increased knowledge of immunology and the importance of the immune response in maintaining the integrity of the organism. Moreover, considerable attention has been focused on the applicability and predictability of laboratory-animal-based assays for immunotoxicity in safety assessment studies (Thomas, 1995). Research in the area of immunotoxicology has established various assays for identifying immunotoxicants, a large array of chemicals known to produce immune effects by either inhibiting or enhancing responses, and specific mechanisms by which some chemicals may produce immune dysfunction. Not all compounds within a given chemical class can act to alter the immune response; however, certain metals, organometals, halogenated hydrocarbons, heterocyclic compounds, organophosphate/chlorine pesticides, and radionuclides can act to alter immune responsiveness of a variety of animal species (including humans) at exposure levels below which other

more commonly utilized endpoints of toxicity are not observed (Luster and Rosenthal, 1993; Luster et al., 1988). Interestingly, many of these same chemicals are found contaminating aquatic environments. Despite the fact that fish may be negatively impacted by these polluting chemicals, little is known regarding the effects of chemicals (either alone or as mixtures) on the fish immune response. In light of increasing social and political pressure to use nonmammalian systems for predicting human health risks and the recent impetus to develop biomarkers for assessing the biological effects of environmental stress, more studies are needed to better understand chemical-induced effects on aquatic species.

Chemical exposure has the potential to interfere with critical phases of the fish immune response, by destroying, sensitizing, or otherwise altering cellular function (e.g., blocking phagocytic activity, inducing or inhibiting cell proliferation, or negating precursor cell formation). Interestingly, many of the same criteria used to determine the immunotoxic potential of xenobiotics in mammalian systems can also be applied successfully to assess immunotoxicity in fish. These include immunopathology, alterations in nonspecific and specific immune functions, and changes in immune-system-regulated parameters (i.e., host-resistance challenge models). In addition, many of the same chemicals that alter immune responses of mammals (including humans) also act as immunotoxicants in fish and in many cases bring about similar effects and appear to act by similar mechanisms (Carlson et al., 2002; Enane et al., 1993; Zelikoff et al., 1991, 2000). To better understand the effects of pollutant chemicals on fish immunocompetence, a battery of immune assays has been employed in such models as tilapia (*Oreochromis niloticus*) (Holladay et al., 1998), rainbow trout (*Oncorhynchus mykiss*) (Cleland et al., 1988), Chinook salmon (*Oncorhynchus tshawytscha*) (Arkoosh et al., 1998), mummichog (*Fundulus heteroclitus*) (Faisal et al., 1991a), turbot (*Scophthalmus maximus*), and Japanese medaka (*Oryzias latipes*) (Beaman et al., 1999). Some of the immune assays more commonly used in fish models include complement fixation, AFC enumeration, respiratory burst activity, phagocytosis, lymphocyte proliferation, mixed lymphocyte response, allograft rejection, delayed hypersensitivity response, cytotoxic T-lymphocytes, serum IgM ELISA, nonspecific cytotoxic cell activity, and host-resistance models. Specific methodologies for these immunoassays have been described elsewhere (Anderson and Zeeman, 1996; Cochet et al., 1998; Stolen and Fletcher, 1994; Stolen et al., 1990; Zelikoff et al., 1996a).

Fish immunotoxicology is a relatively new field of study that is rapidly expanding as more and more techniques and reagents become available for use in teleost species (Anderson and Zeeman, 1996; Gogal et al., 1999; Karol, 1998; Zelikoff, 1998a; Zelikoff et al., 2000). Fish are likely the most well-studied class of aquatic animals in immunotoxicology. Overall, they offer a number of advantages over the current immunotoxicologic mammalian models (e.g., the mouse and rat), including: (1) larger species are available that can provide large numbers of immune cells for study; (2) they are amenable to both laboratory and field studies; (3) they are easily exposed to toxicants in well-defined laboratory situations and under more natural conditions; (4) they are less expensive to buy and maintain than their mammalian counterparts; (5) they offer greater morphological diversity than mammals, thus providing more alternative animal models for study; and (6) they can provide evolutionary reference points for other vertebrate studies. In attempting to make comparisons, however, between fish and mammalian immunotoxicological data, a number of potential difficulties arise, including the lack of bone marrow and lymph nodes in fish, differences in metabolism and pharmacokinetics between species, and limited knowledge regarding interspecies sensitivity to certain chemical toxicants. Despite these, fish models may serve several purposes in bridging the gap between human and ecological risk assessment (Zelikoff, 1994; Zelikoff et al., 2000, 2002). Because fish represent an extremely diverse group of organisms (>25,000 species), it would be anticipated that at least some could serve as a nearly ideal alternate model for investigating immunotoxicity in mammalian species.

Environmental Contaminants

A large number of environmental pollutants are capable of suppressing mammalian immune responses that, under certain circumstances, can lead to increased host susceptibility toward infectious diseases or cancer. Many of these same pollutants also disrupt fish immunocompetence (Beaman et al., 1999; Carlson et al., 2002, 2004; Duffy et al., 2002, 2003; Gogal et al., 1999; Zelikoff, 1994). Stresses imposed on the immune system of fish by environmental pollutants may not always be overtly apparent. Stressors

may act directly to kill the fish or indirectly to exacerbate disease states by lowering resistance and allowing the invasion of environmental pathogens (Zelikoff, 1994). Although the exact relationship between environmental pollution and disease in aquatic organisms is still uncertain, immunosuppression is a strongly supported hypothesis by which aquatic pollutants are thought to increase disease prevalence in exposed fish (Zelikoff, 1993; Zelikoff et al., 1994).

Because a number of comprehensive reviews already exist for immunotoxicological studies carried out between the early 1980s and the mid-1990s (Anderson and Zeeman, 1996; Zelikoff, 1994; Zelikoff et al., 1993), this section focuses only on those studies published since 1994. Results of earlier papers may be considered for comparative purposes or when a particular agent has previously received little attention.

Metals

Contamination of aquatic habitats with heavy metals from various industrial and mineral mining sources has been a problem for many years. The current interests in mineral mining, energy development and use, and dredging will undoubtedly result in further pollution of aquatic environments by such metals as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), and zinc (Zn). The effects of metal pollution are measurable on both ecological and economic scales. Ecosystem impacts include contamination of sediments and the water column, accumulation of pollutants in biota over a wide area, and apparent increases in pollutant-related anomalies in the residing species.

Municipal wastes, industrial discharges, surface runoff, damage and weathering of vessel-protective paints, ocean dumping, and aerial inputs account for most ocean metal pollution. Although some of the routes of entry for metals into the oceans have been slowed or stopped in recent years, too little regulation has been implemented too late. The growing environmental pollution by potentially toxic metals gives rise to particular problems in the aquatic environment. Aquatic organisms' close contact with these metal pollutants and the reactivity of these pollutants cause an accumulation within the organism that is dangerous not only for their own survival but also for humans.

The biological effects of metallic pollutants in aquatic environments are significant (Zelikoff, 1993; Zelikoff et al., 1996b). In addition to alterations in hematological parameters, metabolism, and reproduction and development, laboratory and field studies have demonstrated the ability of pollutant metals to disturb specific immune responses in a variety of fish species. Exposures to certain metals have been shown to alter innate and cell- and humoral-mediated immune functions, as well as interfering with host resistance against infectious pathogens. Although other metals have been studied, this chapter focuses on the immunotoxic effects of cadmium, mercury, copper, chromium, and (organo)tin. For information regarding the effects of other metals on the fish immune response, the reader is referred to reviews by Zelikoff (1993, 1994) and Anderson and Zeeman (1996).

Cadmium

Cadmium (Cd), a known modulator of mammalian immune-defense mechanisms and a commonly occurring environmental contaminant of food, water, and air, represents a major aquatic pollutant in many parts of the world (Brooks and Rumsey, 1974; Sjobeck et al., 1984). *In vivo* studies by Albergoni and Viola (1995a,b) assessing the effects of waterborne Cd exposure on humoral immunity demonstrated that catfish exposed for 7 days to 10, 20, or 30 µg Cd/L (as $CdCl_2$) had significantly reduced titers of total nonspecific Ig; however, levels returned to control values in fish exposed for an additional week. This response may have been due to initial toxicity followed over time by induction of protective enzymes (e.g., metallothionein). Response to a specific antigen was assessed in the aforementioned studies by immunization of Cd-exposed fish with sheep red blood cells (sRBCs). Studies demonstrated that catfish exposed to 20 µg Cd/L required a shorter amount of time than controls to reach peak anti-sRBC IgM levels. Moreover, fish exposed to Cd for 2 weeks prior to immunization reached peak antibody response more quickly and demonstrated a significant increase in antibody titer (compared to controls). Although contradictory findings have been reported (O'Neil, 1981; Robohm, 1986), similar stimulatory effects have been observed in Cd-exposed rainbow trout (*Oncorhynchus mykiss*) following challenge with *Vibrio anguillarum* (Thuvander, 1989) and in metal-exposed striped bass (*Morone saxatilis*) challenged with

Bacillus cereus (Robohm, 1986). Given that the immunomodulatory effects of Cd in mammals as well as fish depends on dose, mode of Cd exposure, and time of exposure in relation to immunization in both fish (Albergoni and Viola, 1995a,b), contradictory results are not surprising.

The effects of Cd on innate immune function represent the best studied area of Cd-induced immunotoxicity in fish (Bennani et al., 1996; Lemaire-Gony et al., 1995; Sanchez-Daron et al., 1999; Voccia et al., 1996; Zelikoff et al., 1994, 1995, 1996b). Studies in this laboratory (Zelikoff et al., 1995) have demonstrated that in the absence of overt toxicity (i.e., changes in total body weight, lysozyme activity, or cell viability) waterborne exposure of mature *Aeromonas salmonicidae*-injected rainbow trout (*Oncorhynchus mykiss*) to Cd at 2 μg/L significantly reduced phorbol myristate acetate (PMA)-stimulated free-radical production (i.e., superoxide [$\cdot O_2^-$] and hydrogen peroxide [H_2O_2] production) after 30 days of exposure. In the same study, phagocytosis was enhanced by Cd exposure after 8 days but returned to control levels after longer exposure durations (17 and 30 days). Carp (*Cyprinus carpio*) exposed to Cd under similar conditions also demonstrated enhanced phagocytic activity (Witeska, 1998); however, unlike that seen in adults, Cd exposure of juvenile trout for 30 days depressed phagocytosis (Sanchez-Dardon et al., 1999; Voccia et al., 1996).

Studies by Zelikoff et al. (1995), Voccia et al. (1996), and Sanchez-Daron et al. (1999) examined the effects of low-dose waterborne Cd exposure on rainbow trout (*Oncorhynchus mykiss*) and demonstrated suppressive effects on PMA-stimulated H_2O_2 production. Intraperitoneal injection of Cd also reduced reactive oxygen intermediate (ROI) production (Bennani et al., 1996). In this study, a single i.p. injection of Cd dose-dependently inhibited bacterially stimulated ROI production by sea bass (*Dicentrarchus labrax*) pronephros phagocytes. On the other hand, *in vitro* treatment of bass phagocytes with Cd had a dose-dependent opposite effect to that observed *in vivo*. It was suggested that discrepancies between the *in vivo* and *in vitro* studies may have been due to differences in the differentiation state of the MØs. Alternatively, effects observed *in vivo* may have been mediated by serum levels of corticosteroids and catecholamines not operative *in vitro*. Inasmuch as Cd acts by inhibiting cellular respiration and uncoupling oxidative phosphorylation in mammalian systems, suppressive effects of Cd on phagocyte ROI production were not unexpected. In contrast to the enhanced effects produced in rainbow trout (Sanchez-Daron et al., 1999; Voccia et al., 1996; Zelikoff et al., 1995) and carp (Bennani et al., 1996), waterborne exposure of Japanese medaka (*Oryzias latipes*) to 6 μg Cd/L for 5 days increased H_2O_2 production compared to control fish (Zelikoff et al., 1996b). Overall, results of the aforementioned *in vivo* and *in vitro* studies demonstrate the sensitivity of fish phagocytes to the immunomodulating effects of Cd. In addition, the findings suggest that ROI production may be the most sensitive indicator of immunotoxic effects associated with exposure to low, environmentally relevant doses of Cd. In fact, its applicability as a biomarker in fish to predict the toxicological impact of contaminated aquatic environments has been suggested (Zelikoff et al., 1994, 1995, 1996b).

In a study examining the effects of metal mixtures on rainbow trout (*Oncorhynchus mykiss*) immunity (Sanchez-Dardon et al., 1999), waterborne exposure for 5 weeks to zinc (30 or 50 μg/L) significantly reduced Cd-induced suppressive effects on MØ phagocytosis and ROI production. Co-exposure to zinc, however, failed to protect against Cd-induced effects on antibody production or B- and T-lymphocyte proliferation. Results of these studies suggest that zinc may act to protect innate immune functions from Cd-induced toxicity in fish by mechanisms similar to those operative in mammals (e.g., induction of metallothionein, prevention of Cd entry into the cell, competition with Cd for intracellular binding sites). The heterogeneity of the results emphasizes the complexity of Cd and the need for further studies to fully understand the mechanisms underlying Cd toxicity in aquatic life.

Although the majority of studies examining the effects of Cd on fish innate immune functions have focused upon MØs, other cells important in maintaining nonspecific host defense also appear to be sensitive targets for Cd-induced immunotoxicity. Studies by Viola et al. (1996), for example, demonstrated that *in vitro* exposure of catfish (*Ictalurus melas*) NCCs to a concentration of soluble Cd as low as 5 μ*M* inhibited their ability to kill human tumor cells; in the presence of 10 μ*M* Cd, cytotoxic activity was completely ameliorated. *In vitro* studies with mammalian NK cells demonstrating the inhibitory effects of Cd on NK cytotoxicity support these findings (Cifone et al., 1990).

Although only a limited number of studies in fish have examined the effects of Cd on cell-mediated immunity, strong evidence exists demonstrating altered T-lymphocyte proliferative responses (Albergoni

and Viola, 1995a,b; Sanchez-Dardon et al., 1999; Thuvander, 1989; Voccia et al., 1996). Voccia et al. (1996) demonstrated that waterborne exposure to Cd at either 1 or 5 ppb depressed proliferation of mitogen-stimulated anterior kidney and thymic lymphocytes. Proliferative responses of thymic lymphocytes were depressed at both concentrations, while lymphoproliferation by kidney cells was reduced only at the highest Cd concentration. Plasma cortisol levels were also reduced in fish exposed to the highest Cd concentration, but the relationship between Cd-induced immunosuppression and depressed cortisol levels in this study remains unclear. In another study performed by the same group of investigators (Sanchez-Dardon et al., 1999), exposure to Cd significantly reduced lipopolysaccharide-stimulated lymphoproliferation. Co-exposure to Zn had no effect on Cd-induced suppression of T-lymphocytes. Moreover, simultaneous exposure of fish to both Cd and mercury inhibited lymphoproliferation to the same degree as exposure to either metal alone. The depressive effects of Cd on T-lymphocyte proliferation have also been observed in catfish (Albergoni and Viola, 1995a,b); neither *in vivo* nor *in vitro* exposure to Cd suppressed PHA-stimulated T-cell proliferation.

Chromium

Chromium (Cr), a highly toxic metal often found in the aquatic environment as the result of spills and other anthropogenic sources, is a potent immunomodulator that poses a substantial health risk to directly exposed organisms. Despite its known immunotoxicity in mammalian systems, few studies have examined the immunotoxic effects of Cr in fish. An *in vivo* study by Khangarot et al. (1999) investigated the effects of exposure for 28 days to subtoxic levels of Cr on host defense against bacterial infection and upon humoral- and cell-mediated immune functions in freshwater catfish (*Saccobranchus fossilis*). In addition to a dose-dependent increase in Cr burdens measured in the kidney, liver, and spleen, Cr exposure significantly altered the spleen-to-body weight ratio, reduced splenic and kidney AFC numbers and antibody titers, altered the numbers and profile of immune cells in the blood and tissues, decreased cell-mediated immunity (as measured by lymphoproliferation and eye-allograft rejection time), and enhanced susceptibility (compared to control fish) to infection with *Aeromonas hydrophila*. In an earlier study using brown trout (*Salmo trutta*) and carp (*Cyprinus carpio*), O'Neill (1981) also demonstrated the suppressive effects of waterborne Cr on humoral immunity. On the other hand, waterborne exposure to hexavalent Cr had no effect on the primary humoral responses of exposed rainbow trout (*Oncorhynchus mykiss*) (Viales and Calamari, 1984). Clearly more studies are necessary to better understand the immunotoxicity of Cr in fish systems.

Copper

Exposure of fish to copper (Cu) may occur in both the natural environment and in aquacultural or aquarium facilities. Copper concentrations may fluctuate in aquatic systems depending on the level of input from wastewater disposal, aquacultural use, marina activity, accidental spills, remediation efforts, or increased surface runoff following storms. In addition, given that several Cu-containing compounds are used as algicides and herbicides in fish food, direct exposure of inhabiting species may occur intentionally. The toxicity of Cu in fish is well documented (Meador, 1991), and Cu-related immunotoxicity has been reported in a number of experimental studies. Results prior to 1994 provided evidence that exposure to Cu suppressed specific antibody responses and phagocyte-mediated ROI production, in addition to increasing the incidence of infectious fish disease.

More recent studies not only support the aforementioned findings but also add to the body of knowledge concerning Cu-induced effects on innate and acquired immunity. In a study by Dethloff and Bailey (1998), rainbow trout (*Oncorhynchus mykiss*) exposed to 6, 16, or 27 µg Cu/L (in soft water) for 3, 7, 14, or 21 days were sacrificed and effects on circulating blood cell profiles, respiratory burst activity, and B- and T-lymphocyte proliferation were examined. Although no correlation was found between hepatic Cu burdens and immune-system alterations, Cu exposure tended to reduce both LPS-stimulated B-lymphocyte proliferation (primarily at that concentration which also reduced cell survival) and head kidney MØ $\cdot O_2^-$ production (for all doses at all time points examined); the proliferation of T-lymphocytes and MØ-mediated phagocytosis was unaffected by Cu exposure. The most consistent findings in this study were effects on blood cell profiles. Percentages of circulating lymphocytes were consistently 9 to

13% below control values, while monocytes were consistently elevated in fish exposed to 27 μg Cu/L. Trout recovered from all three Cu treatment groups (6, 16, and 27 μg Cu/L) demonstrated increased neutrophil levels (compared to control values) with the exception of those fish exposed to 16 μg Cu/L for 7 days. Neutrophil counts from fish exposed to 6, 16, and 27 μg Cu/L for 14 days were increased by 135, 165, and 180% (of control values), respectively. Suppressive effects of Cu on $\cdot O_2^-$ production have been observed in other studies; for example, examination of sea bass (*Dicentrarchus labrax*) 48 hours following a single i.p. injection of Cu demonstrated a dose-dependent reduction of ROI by bacterially stimulated head kidney MØ (Bennani et al., 1996). Moreover, exposure to Cu for 30 minutes *in vitro* had the same immunomodulatory effects on MØ chemiluminescence as that observed *in vivo*. In the same study, i.p. injection of Cu also dose-dependently reduced the phagocytic uptake of live *Aeromonas salmonicidae*.

In contrast to those effects observed in sea bass (*Dicentrarchus labrax*), goldfish (*Carassius auratus*) exposed to waterborne 100 ppb Cu for up to 11 days demonstrated increased intracellular $\cdot O_2^-$ production compared to controls (Jacobson and Reimschuessel, 1998). Furthermore, goldfish exposed to Cu for 4 days and allowed to recover in clean water for 7 days also demonstrated increased $\cdot O_2^-$ production; recovery of fish for only 3 days resulted in a significant reduction in $\cdot O_2^-$ production. To determine whether decreased $\cdot O_2^-$ production was mediated by a humoral factor or a direct effect of Cu on immune function, phagocytes from untreated fish were incubated with serum from either control fish or those allowed to recover for 3 days. *In vitro* exposure of cells to serum from the depurated goldfish resulted in a 29 to 34% decrease in $\cdot O_2^-$ production. It was concluded that decreased $\cdot O_2^-$ production in goldfish recovering 3 days from an acute sublethal Cu exposure was due to an, as of yet, unknown soluble factor present in the serum of recovering animals.

Studies by Rougier et al. (1996) examined the *in vitro* and *in vivo* effects of Cu on zebrafish (*Danio rerio*) nonspecific immune responses. In this study, waterborne exposure of fish for 7 days to 0, 0.05, 0.10, or 0.15 ppm Cu dose-dependently decreased kidney leukocyte number, NCC activity, and phagocytosis of *Aeromonas hydrophila* (as measured by decreased numbers of phagocytically active cells and bacterial numbers within a given cell); suppression of NCC activity (100:1 effector-to-target ratio) and phagocytosis were also observed following *in vitro* exposure of fish kidney cells to 10 or 20 μg Cu per mL. Copper-induced changes in kidney cellularity have also been observed in other fish species (Khangarot and Tripathi, 1991), and it is well known that sublethal levels of Cu have a marked influence on hematological parameters (Christensen et al., 1972; O'Neill, 1981). Although the effects of Cu on immune cell proliferation were not examined, Rougier et al. (1996) suggested that Cu ions might have affected lymphoid organ cellularity by interfering with the replication of immunocompetent kidney cells. These findings could help explain the increased bacterial burdens observed in Cu-exposed zebrafish (*Danio rerio*) following host challenge with *Listeria monocytogenes* (Rougier et al., 1996).

Mercury

Contamination of the aquatic environment by mercury (Hg) has been recognized as a potential environmental and public health problem for over 40 years. It has been estimated that industrial effluents have increased the concentrations of Hg in rivers and lakes by 90 ng/L per year (Wolfe et al., 1998). In aquatic organisms, inorganic Hg can be biotransformed to its most toxic form, methylmercury (Wade et al., 1993). Inorganic Hg has also been shown to damage fish liver and skin (Dalal and Bhattacharya, 1994; Denizeau and Marion, 1990; Sweet and Zelikoff, 2001), as well as arrest gonadal growth and reduce the gonadosomatic index of catfish (Ram and Sathyanesan, 1983).

Although the immunotoxic effects of mercurial compounds have been well studied in mammals (Zelikoff and Cohen, 1997), far less is known concerning the effects in fish (Low and Sin, 1998; MacDougal et al., 1996; Mikryakov and Lapirova, 1997; Sweet and Zelikoff, 2001; Voccia et al., 1994; Zelikoff et al., 1996). Immunotoxic effects following Hg exposure range from depressed hematopoiesis and enzyme activity to enhanced immune cell death. Investigators have observed a continuum of effects ranging from low-dose activation to high-dose inhibition of fish immune cell function following Hg exposure (Low and Sin, 1998; MacDougal et al., 1996; Voccia et al., 1994). Relatively low concentrations (e.g., 0.1 to 1 μ*M*) of Hg have been shown to increase lymphocyte mitosis and intracellular calcium ($[Ca]_i$) levels, whereas higher concentrations suppress DNA synthesis, induce rapid Ca influx, and alter

tyrosine phosphorylation of cell proteins (Low and Sin, 1998; MacDougal et al., 1996). Head kidney MØs recovered from rainbow trout (*Oncorhynchus mykiss*) exposed for several weeks to 0.5 ppb Hg exhibited diminished phagocytosis, respiratory burst activity, and Ig levels (Sanchez-Dardon et al., 1999). Japanese medaka (*Oryzias latipes*) exposed to a tenfold higher Hg concentration for 5 days failed to exhibit similar responses (Zelikoff et al., 1998).

Similar to that seen in trout, exposure to divalent Hg also alters the immune system of blue gourami (*Trichogaster trichopterus*) (Low and Sin, 1998). Exposure of fish for 2 weeks to 0.09 ppm Hg significantly increased gourami kidney lysozyme activity. Interestingly, enzyme activity decreased to control levels following co-exposure to an equimolar concentration of selenium (as SeO_3^{2-}). Other studies by the same authors demonstrated similar findings in tilapia (*Oreochromis*). In this study, fish exposed to 0.01 mg Hg per L had significantly reduced plasma agglutinating antibody titers 7 and 8 weeks following immunization with formalin-killed *Aeromonas hydrophila*; waterborne exposure to 0.09 mg Hg per L reduced agglutinating titers for 5 weeks after infection. Tilapia, co-exposed to Hg plus 0.5 mg SeO_3^{2-} per L, demonstrated significantly decreased agglutinating antibody titers in the first 3 weeks following bacterial injection. Overall, effects of Hg on fish agglutination titers were inconsistent and difficult to interpret. *In vitro* studies have also been carried out to determine Hg-induced effects on lymphocyte proliferation. In the presence of either 0.09 or 0.18 mg Hg per L, mitosis of Con A-stimulated T-lymphocytes was significantly inhibited, while incubation with a lower Hg dose (0.04 mg Hg per L) significantly enhanced the mitotic rate. Taken together, the aforementioned studies support the hypothesis that immunotoxic doses of Hg inappropriately alter fish immunity, thus modifying the ability to regulate the magnitude and specificity of a competent immune response.

Tin

Although some inorganic tin (Sn) compounds have demonstrated toxicity, it is the synthesized organotins (with the potential for persistence in aquatic environments) that pose the greatest threat to teleost and mammalian species (O'Halloran et al., 1998; Zelikoff, 1993; Zelikoff an Cohen, 1997). Organotins are used in a variety of products, including as stabilizers in polyvinyl chloride, catalysts in polyurethane and silicone elastomers, and pesticides (Grinwis et al., 1998). The best studied of the organotins, tribuyltin (TBT) is most commonly used in antifouling paints for small ships. Although banned in many countries (including the United States), TBT can still be found in concentrations as high as 5 ppb in some Canadian freshwaters (Maguire et al., 1986), 1.5 ppb in French marine waters (Alzieu et al., 1989), and 7 ppb in some Netherlands harbors (Ritsema and Laane, 1991). Moreover, fish have been shown to bioaccumulate organotins by two to three orders of magnitude. In addition to their known hepatotoxicity and effects on the endocrine system (Snoeij et al., 1985), organotins are well-known mammalian immunotoxicants. Exposure to organotin compounds has been shown in mammals to reduce cellularity of the thymus and spleen, circulating and splenic lymphocyte numbers, T-lymphocyte-dependent immunity, host resistance against infectious agents, and tumoricidal activity (Zelikoff and Cohen, 1997).

Prior to 1994, most studies assessing the immunotoxicity of organotin compounds in fish were performed *in vitro* (Rice and Weeks, 1989; Wishovsky et al., 1989). In one study, exposure to TBT reduced phagocyte-mediated immune functions in a time- and dose-dependent manner (Rice and Weeks, 1989). In a more recent *in vitro* investigation, O'Halloran et al. (1998) examined the effects of TBT and its metabolite dibutyltin (DBT) on immune cells isolated from the spleen and head kidney of juvenile rainbow trout (*Oncorhynchus mykiss*). Immune function was unaltered by exposure to the lowest dose of either organotin compound (i.e., 2.5 µg/L), but incubation of lymphocytes with 50 µg DBT/L significantly depressed mitogen-stimulated proliferation. Mitogenesis of LPS-stimulated splenic and kidney B-lymphocytes was also reduced by the same DBT concentration. Changes in the cell population profile appear to be responsible for the DBT-induced inhibition of lymphocyte proliferation. In contrast, NCC activity was unaffected by *in vitro* exposure to either organotin compound. It was suggested that organotins might exhibit both functional and tissue-specific effects on the fish immune system (i.e., spleen > head kidney, and, in general, LPS responsivity > Con A-responsive leukocytes). Based on the observed results, the authors questioned whether TBT did, indeed, represent the most toxic form of organotin.

In another study, Grinwis et al. (1998) examined the short-term toxicity of *bis*(tri-*n*-butyltin)oxide (TBTO) on exposed flounder (*Platichthys flesus*) with particular emphasis on histology and immune function. Although exposure of flounder for 6 days to TBTO reduced thymus volume (related to body weight), circulating lymphocyte values, total splenic lymphocyte numbers, and NCC activity, these effects occurred only at a TBTO concentration that resulted in high mortality and extensive histopathological lesions throughout the body (i.e., 32 μg TBTO per L). With some exceptions, effects in flounder appear to contrast with those observed in TBT-exposed medaka, guppies, or rainbow trout.

Taken together, it appears that exposure to a variety of contaminant metals can alter the immunologic competence of exposed fish. Metal-induced immune alterations appear to occur either directly by binding to, or readjusting, the tertiary structures of biologically active molecules or indirectly by acting as stressors to modify corticosteroid levels in fish. Regardless of the mechanisms by which effects may occur, however, a metal-induced reduction in innate, cell-mediated, or humoral immunity can lead to suppressed immune function followed, ultimately, by increased susceptibility of fish to infectious diseases or cancer.

Pesticides

Pesticides represent a diverse group of poisonous chemicals that are primarily used to control pest species of insects, weeds, or fungi. Many of the more persistent pesticides, especially the chlorinated hydrocarbons, are common pollutants of aquatic ecosystems. Pesticides enter the aquatic environment and become rapidly distributed through intentional application, aerial drift, runoff from agricultural fields following application, or accidental release. Fish residing in contaminated areas frequently encounter and accumulate sublethal levels of pesticides from the surrounding water and food, and they may then enter the food chain. In addition to the carcinogenic potential of some pesticides and their toxicity for the nervous and reproductive systems (Klaassen et al., 1986), pesticides represent a potential immunotoxic threat to all directly exposed organisms. Yet, despite this and their potential presence in the aquatic environment, relatively few studies have examined the toxic effects of pesticides on the immune response of fish.

Organophosphate Insecticides

Organophosphorus insecticides, discovered in Germany just before World War II, have become the most widely used insecticides and have replaced organochlorines as they eliminate problems associated with environmental persistence, bioconcentration, and biomagnification. Inhibition of acetylcholinesterase (AChE) is considered to be the basis for organophosphate (OP) insecticide toxicity (Albers et al., 1999), but mammalian literature also provides strong evidence for their immunosuppressive effects (Barnett and Rodgers, 1994). It remains less clear as to what extent nonmammalian species (in particular aquatic organisms) may be impacted by these chemicals. Most early studies in fish examined the immunomodulating effects of trichlorphon and malathion (Areechon and Plumb, 1990; Cossarini-Dunier et al., 1990; Dutta et al., 1992; Mishra and Srivastava, 1983; Plumb and Areechon, 1990; Siwiki et al., 1990). Although somewhat limited in nature, these studies provided the groundwork for demonstrating the immunosuppressive effects of OP insecticides in fish. Malathion continues to be examined (Beaman et al., 1999) along with some less well-studied OP insecticides such as chlorpyrifos, edifenphos, and glyphosate.

Malathion—Diethyl(dimethoxyphosphinothioylthio)succinate (malathion) is a wide-spectrum OP insecticide used worldwide to control fruit- and vegetable-sucking and chewing insects, disease vectors such as mosquitoes and flies, and ectoparasites of various animal species (Barnett and Rodgers, 1994). The most well-studied effects of malathion are its ability to inhibit AChE activity (likely through the binding of malaxon to the AchE receptor) (Shao-nan and De-fang, 1996) and disrupt nerve impulse transmission (Richmonds and Dutta, 1992), but malathion has also been shown to modify mammalian host-defense mechanisms (Barnett and Rodgers, 1994). Although studies are limited, malathion appears to affect the immune system of fish in a manner similar to that seen in mammals (Areechon and Plumb, 1990; Beaman et al., 1999; Dutta et al., 1992; Mishra and Srivastava, 1983; Plumb and Areechon, 1990). Studies by Beaman et al. (1999) demonstrated that exposure of Japanese medaka (*Oryzias latipes*) for 7 or 14 days to sublethal concentrations of malathion (0.2 or 0.8 ppm) dose-dependently depressed T-dependent AFC

numbers. Changes in humoral immunity occurred in the absence of effects on hematocrit/leukocrit or T-lymphocyte proliferative responses. Malathion has also been shown to reduce antibody titers against *Edwardsiella ictaluri* in channel catfish (*Ictalurus punctatus*) (Plumb and Areechon, 1990). These studies also demonstrated that a 14- or 21-day exposure of medaka to either 0.1 or 0.3 ppm malathion significantly reduced host resistance against *Yersinia ruckeri* infection. Although studies indicate that malathion-induced toxicity in fish may not be mediated by the same detoxification enzymes important for bringing about effects in mammals (Pathiratne and George, 1998), it appears that mammalian immune assays can be used successfully in a teleost model for predicting malathion-induced immunosuppression.

Chlorpyrifos—Chlorpyrifos, developed in 1962 to control a wide variety of agricultural- and urban-associated insects, has wide application as a termiticide (Albers et al., 1999). The health effects of chlorpyrifos on exposed humans is highly controversial, although it appears that relatively high doses are needed before any effects are observed. In addition to neurological outcomes, a few clinical and epidemiological studies have suggested that exposure to chlorpyrifos can alter hematologic and immunologic parameters in exposed individuals (Broughton et al., 1990; Fiedler et al., 1992; Thrasher et al., 1993). Chlorpyrifos has been found to contaminate aquatic environments (usually via runoff from treated sites), but very few studies have examined the effects of this OP insecticide on the immune response of fish. In a study by Holladay et al. (1996), tilapia (*Oreochromis niloticus*) exposed in the water to 1 ppb chlorpyrifos demonstrated reduced pronephros cellularity and depressed phagocytic function (compared to controls) in the absence of effects on host mortality, body weight, or oxidative burst activity.

Edifenphos and Glyphosate—In a study by El-Gendy et al. (1998), Bolti fish (*Tilapia nilotica*) were exposed for 96 hours to edifenphos or glyphosate, and effects on protein patterns and cellular and humoral immune responses were investigated at different time intervals after the exposure. Control values were poorly defined, but it appears that mitogen-stimulated lymphocyte proliferation decreased significantly in treated fish (compared to control); maximal depression was reached 4 weeks following exposure. Moreover, exposure of fish to edifenphos and glyphosate reduced splenic AFC numbers and mean serum antibody titers; effects on humoral immunity are similar to those produced by other OP insecticides, including malathion (Beaman et al., 1999). Because ACh is increased as a result of OP-induced inhibition of AchE, suppressive effects of edifenphos and glyphosate may have been due to the direct action of acetylcholine (ACh) on the immune system.

Pyrethroids

Pyrethroids are pharmacologically active synthetic compounds structurally based on the naturally occurring substance pyrethrum, which is derived from chrysanthemum flower extracts. The recently introduced synthetic pyrethroids (i.e., *S*-biallethrin, permethrin, cysmethrin, biosresmethrin, cypermethrin, deltamethrin, and fenvalerate) have attracted wide use by farmers primarily because of their potent insecticidal nature and apparent lack of human toxicity; because synthetic pyrethroids are neither fully metabolized nor quickly detoxified, they have the potential to create a serious residue accumulation problem (Desi et al., 1986). Moreover, it has been suggested that covalent binding of the reactive pyrethroids to liver proteins can create new haptens and potent antigens, resulting in hypersensitivity reactions (Diel et al., 1998; Hoellinger et al., 1987). Immunosuppressive effects of some synthetic pyrethroids have also been described (Desi et al., 1986). In a study by Madsen et al. (1996), rats exposed for 28 days by gavage to deltamethrin had increased mesenterial lymph node weight, decreased thymus weight, and increased AFC numbers and splenic NK cell activity; similar effects were not observed following exposure to α-cypermethrin. Other pyrethroids have also been shown to alter the production of cytokines in human lymphocyte cultures (Diel et al., 1999).

Although the new generation of pyrethroids appears to pose a limited threat to humans, they are for the most part highly toxic to fish (Agnihothrudu, 1988; Bradbury et al., 1985). Exposure of *Cyprinus carpio* eggs to increasing concentrations of cypermethrin, deltamethrin, or fenvalerate resulted in reduced hatching ability and viable hatch, as well as inactive or abnormal larvae (i.e., increased vertebral column flexure, enlarged yolk and pericardial sacs, and stunted tail). The majority of laboratory studies examining the effects of synthetic pyrethroids on fish have focused on a single member of the newest classes of

type I pyrethroids, permethrin. Since its synthesis in 1973, permethrin has gained in popularity due to its photostability, low mammalian and avian toxicity, and relatively short environmental half-life (i.e., a half-life of 28 days in soil and 14 days in seawater exposed to sunlight) (Rebach, 1999). Unfortunately, even though permethrin appears to degrade rapidly in water, it tends to bioconcentrate in fish (Wei et al., 1995). In fact, the major contraindication to permethrin use is its relatively high toxicity to non-target organisms, including macro-invertebrates (ranging from 0.02 to 0.73 ppb in species tested) and estuarine fishes (ranging from 2.2 to 12 ppb in species tested).

In addition to acute toxicity (Hohreiter et al., 1991; Holcombe et al., 1982; Rebach, 1999) and neurological effects (i.e., restlessness, loss of coordination, systemic tremors, and paralysis) (Eells et al., 1993; Rebach, 1999), several studies have shown that exposure of fish to permethrin also modulates immune function (Sopinska and Guz, 1998; Zelikoff et al., 1997). Exposure of carp (*Cyprinus carpio*) for example, for 96 hours to the synthetic pyrethroid Ambusz 25EC (at a concentration of 0.03 or 1.1 ppb) induced leukopenia, neutrocytosis, and reduced the percentage of kidney cells active in phagocytosis (Sopinska and Guz, 1998). Given that shorter and longer exposure times failed to produce similar effects, however, the results of these studies are questionable. Zelikoff et al. (1998) examined the immunomodulating effects of permethrin at concentrations ranging from 0.01 to 2.0 ppb on Japanese medaka (*Oryzias latipes*). Although no effects were observed on body or immune organ weight, kidney and spleen cell viability, or hematocrit and leukocrit levels, permethrin concentrations ≥0.5 ppb tended to reduce plasma Ig levels and kidney and spleen cellularity after 7 days (compared to solvent-exposed controls); thymic cellularity was reduced by about 30% in fish exposed to the highest permethrin concentration. Alternatively, exposure to permethrin for 48 hours tended to increase the proliferative response of splenic lymphocytes. Probably the most dramatic effect of permethrin exposure in this study was on host survival following bacterial challenge. Exposure of medaka to either 0.05 or 0.01 ppb permethrin for 2 or 7 days, respectively, increased host mortality by about 30% in response to *Yersinia ruckeri* infection (compared to unexposed, bacterially challenged fish). Results in medaka resemble those produced in BALBc mice exposed to permethrin by oral gavage (Blaylock et al., 1995). In the latter case, exposure to 1.0, 0.1, or 0.01% permethrin for 10 days had no effect on body and spleen weight or mitogen-stimulated lymphocyte proliferation. Alternatively, those responses in mice requiring specific antigen recognition or effector function (i.e., MLR, CTL, or NK activity) were depressed by permethrin exposure.

Organochlorine Insecticides

The organochlorines (OCs), popular for their potent insecticidal activity and cost efficiency, consist of three major families of compounds: (1) those related to DDT, including dicofol and methoxychlor; (2) cyclodienes, such as aldrin and dieldrin; and (3) hexachlorocyclohexanes, such as lindane. To varying degrees, OCs are all characterized by low water solubility and high chemical stability that contribute to their persistence in the environment. The highly lipophilic nature of these compounds makes them subject to bioaccumulation such that organisms of sequentially higher trophic levels concentrate the pesticides from surrounding soil and water or through the consumption of organisms in lower trophic levels. In mammals, the most commonly observed non-immune effects of OC pesticides relate to hepatotoxicity and neurotoxicity with some difference in the actual symptoms produced by a given agent or isomer of that agent (Barnett and Rodgers, 1994).

Lindane—Five isomers of 1,2,3,4,5,6-hexachlorocyclohexane (HCH)—alpha (α), beta (β), gamma (γ), delta (δ), and epsilon (ε)—were originally contained in a commercial preparation of HCH known as benzene hexachloride. This compound has not been marketed since 1978, but OC γ-HCH is commercially available as the insecticide lindane. Lindane is used as an insecticide treatment for crop protection and in human and veterinary medicine against ectoparasites. Excessive lindane can be persistent in food chains and readily accumulated by most animal species; fish can absorb lindane directly from the water or by ingestion of contaminated food, and it can bioaccumulate in fish at ratios of 500- to 1200-fold (Betoulle et al., 2000). In addition to reported effects on hematological parameters (Ferrando and Andreu-Moliner, 1991), ATPase activity (Hanke et al., 1983), and nervous system function (Joy, 1982), a number of studies have demonstrated the immunomodulatory effects of lindane in fish (Betoulle et al., 2000; Ferrando and Andreu-Moliner, 1991; Hart et al., 1997; Svensson et al., 1994; Sweet et al., 1998). Early

studies demonstrated that i.p. injection of 10, 50, or 100 mg lindane per kg BW significantly depresses cell-, humoral-, and nonspecific immune responses of rainbow trout (*Oncorhynchus mykiss*) (Cossarini-Dunier, 1987; Dunier and Siwicki, 1994; Dunier et al., 1994); similar effects were not observed in lindane-exposed carp (*Cyprinus carpio*) (Cossarini-Dunier, 1987). More recent investigations demonstrated that i.p. injection of tilapia (*Oreochromis niloticus*) with 20 or 40 mg lindane per kg BW for 5 consecutive days dose-dependently reduced splenic and pronephric white blood cell (WBC) counts (Hart et al., 1997). Hypocellularity, determined from histological examination, was also observed in these same lymphoid tissues. In contrast, exposure of fish to the same lindane concentration had no effect on phagocyte function. Because exposure to α, β, γ, and δ isomers of HCH (concentrations ranging between 10 and 100 μM) have been shown *in vitro* to induce apoptosis in lake trout (*Salvelinus namaycush*) thymocytes (Sweet et al., 1998), the lymphoid organ hypocellularity in tilapia may have been a result of enhanced apoptotic cell death. The authors of this study concluded that lymphocytes represent a more sensitive cell type to the effects of lindane than those associated with innate immunity. Using an exposure route and lindane concentrations similar to those employed for the tilapia studies, Dunier et al. (1994) also demonstrated lymphocytes to be a sensitive target for lindane exposure. In an *in vitro* study by Betoulle et al. (2000), incubation of rainbow trout (*Oncorhynchus mykiss*) phagocytic cells with lindane concentrations between 50 and 200 μM induced excessive cell mortality. Cell death appeared to be related to increased ROI production and $[Ca^{2+}]_i$ levels. Based on these findings, a second *in vitro* study was performed in which rainbow trout head kidney phagocytes and peripheral blood leukocytes were exposed to lower lindane concentrations between 2.5 and 100 μM and effects upon $[Ca^{+2}]_i$ levels examined. Peripheral blood lymphocytes exposed to lindane concentrations between 5 and 100 μM demonstrated increased $[Ca^{+2}]_i$ levels, as did phagocytes exposed to lindane concentrations ≥ 25 μM. In phagocytes, lindane required higher extracellular calcium $[Ca^{+2}]_e$ levels to raise $[Ca^{+2}]_i$.

Halogenated Aromatic Hydrocarbons

Halogenated aromatic hydrocarbons (HAHs) are low-molecular-weight compounds that are classified into several major families: polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polychlorinated terphenyls (PCTs), polychlorinated naphthalenes (PCNs), and polychlorinated diphenyl ethers (PDEs). In recent years, halogenated aromatic compounds have engendered increasing concern about their impact as environmental pollutants. The physical and chemical stability of the higher chlorinated compounds along with their lipophilicity contribute to their nearly ubiquitous occurrence and to efficient bioaccumulation via the aquatic and terrestrial food chains. Polychlorinated biphenyls have appeared as frequent contaminants of soil and water, and chlorophenols have been detected in surface and drinking water. The extremely toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) has contaminated large areas of both water and soil through industrial accidents, improper waste disposal, and wide-scale application of herbicides containing small quantities of the chemical as a contaminant, in addition to being a trace byproduct of combustion. Few data exist concerning the effects of PCTs, PCNs, and PDEs on the immune response, but the mammalian literature is replete with studies demonstrating the immunotoxicity of PCDDs, PCDFs, and PCBs (Holsapple, 1996).

2,3,7,8-Tetrachlorodibenzo-p-Dioxin

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the most biologically potent of the HAHs. Exposure to TCDD (and structurally related HAHs) produces a number of toxic effects in laboratory mammals, including a generalized wasting syndrome, lymphoid involution (especially of the thymus), pancytopenia, immunosuppression, hepatomegaly and hepatoxicity chloracne, hyperkeratosis, gastric lesions, urinary tract hyperplasia, edema, tumor promotion, teratogenicity, and decreased spermatogenesis (Holsapple, 1996). In addition to the potency differences associated with the structure–activity relationship, HAH-induced toxicity is characterized by wide variations in potency among different species of laboratory animals; for example, the LD_{50} for TCDD varies approximately 5000-fold between the most sensitive (guinea pigs, 0.6 to 2 $\mu g/kg$) and the least sensitive (hamsters, 5 mg/kg) species. Although the spectrum of toxicity can vary markedly among species, certain toxic effects have been observed as correlates to

exposure in almost all species tested. The most notable of these effects are the generalized slow-wasting syndrome, thymic atrophy, immunosuppression, and hepatomegaly. In fact, the observation that lymphoid involution is among the earliest and most sensitive manifestations of TCDD (and related HAH) exposure and that it is almost universally observed among animal species provided the impetus to characterize HAH immunotoxicity.

Unbelievably few studies have been performed examining the effects of dioxin on the immune responses of fish, despite the common occurrence of TCDDs in the aquatic environment; potential accumulation in the food chain; effects on fish growth, survival, and reproduction (Giesy et al., 2002); and demonstrated immunotoxicity in multiple mammalian species (Dearstyne and Kerkvliet, 2002; Holsapple, 1996; Kerkvliet and Burleson, 1994). Most of those studies, however, have demonstrated TCDD-induced lymphoid organ hypocellularity (Grinwis et al., 2000) and, in some cases, thymic involution (Spitsbergen et al., 1988). In a study employing two different strains of juvenile rainbow trout (Shasta and Wytheville), Spitsbergen et al. (1988) demonstrated that i.p. injection of fish with 1 μg TCDD per kg produced numerous lymphomyeloid lesions, including thymic involution, splenic lymphoid depletion, and hypocellularity of kidney hematopoietic tissues. In association with decreased hematopoiesis, Shasta strain yearling trout also demonstrated peripheral leukopenia and thrombocytopenia. It was concluded that lymphomyeloid and epithelial tissues were the primary targets for TCDD-induced pathologic lesions in rainbow trout (*Oncorhynchus mykiss*) and that lesion prevalence and severity were influenced by trout strain and hatchery source. In another study performed by the same laboratory, yearling trout were injected i.p. with up to 10 μg TCDD per kg, and the effects on prefrontal cortex (PFC) numbers, MØ-mediated phagocytosis, and mitogen-stimulated blastogenesis of thymic and splenic lymphocytes were evaluated (Spitsbergen et al., 1986). At the highest TCDD dose, fish exhibited a variety of morphologic and histologic alterations but few changes in immune function (with the exception of reduced pokeweed mitogen-induced splenocyte proliferation). It was concluded that although trout appeared to be among the more sensitive of species with regard to lethal effects, they were relatively resistant to the immunosuppressive potential of TCDD.

Even though host resistance against viral infection is considered to be an extremely sensitive endpoint for TCDD-induced immunotoxicity in mammals (TCDD-induced effects occurring at nanogram concentrations) (Nohara et al., 2002), trout fingerlings fed a diet of ≤1 μg TCDD and subsequently challenged with infectious hematopoietic necrosis virus had no change in disease resistance compared to control fish (Spitsbergen et al., 1988). The general failure of TCDD to suppress rainbow trout (*Oncorhynchus mykiss*) immune function at doses below those causing clinical toxicity parallels findings with other pharmacologic immunomodulators (Sakai et al., 2000). Studies performed with teleost species other than trout also demonstrate the sensitivity of lymphomyeloid tissue to dioxin exposure, as well as the relative resistance of other fish species to TCDD-induced immunosuppression; for example, i.p. exposure of yellow perch (*Perca flavescens*) to 5 μg TCDD per kg produced a variety of histologic pathologies, including decreased splenic cellularity. Thymic atrophy and decreased head kidney hematopoiesis (among other histologic and morphologic lesions) were also observed in this study, but only at TCDD doses producing 95% mortality (25 and 125 μg TCDD/kg). In a more recent study, repeated dioxin exposure (i.p. injection of either 1 or 5 μg/kg/day for a total of 5 days) of tilapia (*Oreochromis niloticus*) reduced lymphoid cellularity (Hart et al., 1999). Finally, Grinwis et al. (2000) demonstrated that oral exposure of European flounder (*Platichthys flesus*) to dioxin (at a 50-fold higher dose than that needed to produce acute toxicity in trout—500 μg/kg), increased mitotic activity and hepatosomatic index, but only slightly reduced thymus size. These studies demonstrate that fish (like their mammalian counterparts) vary dramatically in their overall sensitivity to dioxin.

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) exist as major contaminants in water resources worldwide (Anderson et al., 2003; Barron et al., 2000). Barron et al. (2000) demonstrated that whole body and liver samples from adult walleye (*Stizostedion vitreum vitreum*) collected from the Lower Fox River and Green Bay, Wisconsin, contained 4.6 to 8.6 and 4.1 to 7.9 mg total PCB per kg wet weight, respectively (compared to 0.04 mg PCB per kg in reference fish). Although some PCB contamination in the environment has resulted from commercial use, most is due to careless disposal practices or leakage

from industrial facilities or chemical waste disposal sites (Segre et al., 2002). The 209 PCB congeners are divided into three classes based on positioning of the chlorine molecules—namely, coplanar, mono-ortho coplanar, and noncoplanar. The number of chlorine molecules determines the solubility and therefore persistence of each congener in water. As the chlorine content of the congener increases, the congener becomes less water soluble (Swain, 1991). Coplanar congeners appear to have the greatest potential for toxicity, as well as the greatest affinity for the aryl hydrocarbon receptor (AhR). Acute exposure to PCBs, most often through contaminated foodstuffs (particularly fatty fish species), has been associated with (or shown to cause) a variety of adverse effects in exposed hosts, including altered physiological and biochemical responses, anatomical deformities, suppressed immunocompetence, and, possibly, cancer (Svensson et al., 1994). Long-term PCB exposure has been shown to cause reproductive, neurologic, and genetic toxicity both in humans and laboratory-exposed rodents (Lee and Chang, 1985).

Experimental studies in rodents and nonhuman primates exposed to PCBs have demonstrated that the immune system is perhaps the most sensitive target for PCB-induced toxicity (Davis and Safe, 1990; Omara et al., 1998; Smithwick et al., 2001). In humans, individual PCBs (or their mixtures) consistently produce thymic and splenic atrophy, bone marrow depression, and loss of lymph-node germinal centers (Tryphonas and Feeley, 2001). PCB-induced lymphoid atrophy, suppressed immune function (i.e., antibody titers, PFC numbers, NK cell activity, T-lymphocyte proliferation, and graft-vs.-host reaction), and host resistance against infectious bacterial and viral pathogens (i.e., *Listeria monocytogenes*, Moloney leukemia virus, herpes simplex virus, *Plasmodium berghei*, and *Salmonella typhosa* and *S. typhimurium*) have been demonstrated in laboratory-exposed rodents (Tryphonas, 1995). Moreover, prenatal PCB exposure also seems to affect host immunocompetence (Wu et al., 1999; Segre et al., 2002). For example, 4- and 6-week-old offspring birthed from dams exposed immediately before mating to a single dose of Arochlor® 1254 (300 mg/kg) demonstrated reduced thymic weight (particularly in the younger age group) and altered immune responses (Wu et al., 1999). Although the mechanisms by which PCBs induce their immunotoxic effects are still being considered, some of the coplanar congener-induced effects seem to be mediated by AhR binding in the lymphoid organs and immunocompetent cells.

Fish, like their mammalian counterparts, also appear sensitive to the immunotoxic effects of PCBs (Anderson and Zeeman, 1996). Despite some inconsistencies between studies, exposure to PCBs frequently produces tissue atrophy (Grinwis et al., 2001), suppressed immune function, and reduced host resistance in exposed fish (Arkoosh et al., 1993; Burton et al., 2002; Duffy et al., 2002, 2003; Jones et al., 1979; Regala et al., 2001; Rice and Schlenk, 1995; Thuvander et al., 1993). In a study by Grinwis et al. (2001), European flounder (*Platichthys flesus*) exposed orally to 0, 0.5, 5, or 50 mg PCB 126 per kg BW demonstrated reduced thymus size, but only at the highest exposure dose. It was speculated that such thymic atrophy could underlie the observed increase of infectious disease (i.e., viral lymphocystis) recently observed in feral flounder populations. The authors concluded that effects produced by PCB 126 were actually more dramatic than those reported previously for TCDD. This suggests that the toxic equivalency factor (TEF) of 0.0005 assigned to PCB 126 from early life stage mortality studies in rainbow trout (*Oncorhynchus mykiss*) might underestimate the actual toxic potential of PCB 126. In another study, i.p. injection of channel catfish (*Ictalurus punctatus*) with 0.01, 0.1, or 1.0 mg PCB 126 per kg BW reduced NCC activity and phagocyte-mediated oxidative burst, while increasing antigen-specific AFC numbers 3, 7, and 14 days following exposure (Rice and Schlenk, 1995). In a more recent study employing the same fish species and PCB 126 doses, oxidative burst activity was depressed (compared to controls) for up to 14 days in fish treated with the highest PCB 126 dose and as long as 21 days following exposure to the lowest PCB dose (Regala et al., 2001). Other laboratory studies have demonstrated that exposure of fish to PCBs (either as a single congener or Arochlor® mixture) reduces phagocytic activity (Jones et al., 1979), stimulates both T- and B-lymphocyte proliferation in response to mitogens (Thuvander and Carlstein, 1991), and depresses both the primary and secondary AFC response (Arkoosh et al., 1994). In this laboratory, a single i.p. injection of Japanese medaka (*Oryzias latipes*) with either 0.1 or 1.0 µg PCB 126 per g BW significantly reduced (compared to vehicle control) AFC numbers and increased intracellular $\cdot O_2^-$ production in an age- and concentration-dependent manner (Duffy et al., 2002. 2003). In some cases, co-exposure to other environmentally relevant contaminants appeared to potentiate PCB-induced

effects (Burton et al., 2002). Although differences between fish species in the magnitude or degree of PCB-induced immunotoxicity may exist, that PCBs can modulate immune responses in fish is becoming well established.

Host resistance against infectious disease challenge is by far the most well-studied immune endpoint used to assess PCB-induced effects in fish (Arkoosh et al., 1998; Jones, 1979; Mayer et al., 1977; Snarski, 1982). Although the effects appear dependent on fish species, challenge organism, and fish age at the time of pathogen challenge, host resistance against infectious-disease-producing pathogens appears to be decreased by PCB exposure. Rainbow trout (*Oncorhynchus mykiss*) exposed to waste mixtures of PCBs demonstrated decreased resistance to yersiniosis (Mayer et al., 1977), and PCB-exposed channel catfish (*Ictalurus punctatus*) demonstrated increased disease susceptibility to infection with *Aeromonas hydrophila* (Jones et al., 1979).

Field investigations of PCB-exposed feral fish populations have demonstrated effects similar to those seen in controlled laboratory studies (Anderson et al., 1997, 2003; Arkoosh et al., 1998; Barron et al., 2000). Chinook salmon, for example, recovered from a PCB/PAH-contaminated estuary of Puget Sound in Washington state, and subsequent challenge with *V. anguillarum* produced a higher cumulative mortality after pathogen exposure than in salmon from hatcheries or a non-urban estuary (Arkoosh et. al., 1998). In another study, smallmouth bass (*Micropterus dolomieu*) from either a reference or PCB-contaminated site in the Great Lakes were examined, and the effects on a variety of biochemical, histological, and immunological parameters were determined (Anderson et al., 1997, 2003). Fish from the impacted site appeared severely immunocompromised compared to the reference-site fish. Although hematocrit/leukocrit and total plasma Ig levels remained unchanged, superoxide dismutase (SOD) and immune functional activities such as MØ-mediated phagocytosis and $\cdot O_2^-$ production were significantly reduced in fish recovered from the PCB-impacted site (compared to reference-site bass). Moreover, examination in tandem with other endpoints of injury demonstrated the enhanced sensitivity, applicability, and reproducibility of immune assays for demonstrating exposure to—and biological effects from—chemically polluted aquatic environments. Lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*) recovered from a PCB-contaminated Great Lakes site near the area from which the smallmouth bass were collected (Anderson et al., 1997, 2003) also demonstrated substantial immunological injury. Changes in circulating blood cell profiles seemed to be the most obvious effect in the PCB-exposed lake trout (i.e., increased percentages of neutrophils and monocytes and decreased lymphocyte levels), but the brown trout demonstrated increased levels of circulating WBC numbers and total plasma Ig values. The exposed brown trout also demonstrated reduced phagocytic activity and $\cdot O_2^-$ production compared to reference-site fish. Superoxide dismutase activity was diminished in both PCB-exposed trout species. Results from the aforementioned field studies along with those examining walleye (*Stizostedion vitreum vitreum*) also captured from a nearby PCB-impacted site (Barron et al., 2000) demonstrate the utility of immune assays for assessing PCB-induced toxicity in exposed feral fish populations.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), well known for their carcinogenic and mutagenic actions, readily enter the environment via incomplete combustion of organic matter. Because individuals can be exposed to significant amounts in the atmosphere, soil, waterways and oceans, and the food chain, PAHs represent a serious health concern for humans (Ladics and White, 1996). The primary sources of PAHs in the atmosphere are from transportation emissions, coal- and oil-fired power stations, domestic heating, cigarette smoke, refuse burning, forest fires, volcanic activity, and industrial processes (i.e., petroleum products and tar). Baum (1978) reported that approximately 900 tons of benzo(*a*)pyrene (BaP) are released into the U.S. air annually. From the atmosphere, PAH compounds are dispersed into water systems, soil, and marine biota.

In addition to their carcinogenic and mutagenic activities (White et al., 1994), many PAHs are also potent immunosuppressants (Ladics and White, 1996). The most toxic and best studied PAHs are BaP, 7,12-dimethylbenz(*a*)anthracene (DMBA), and 3-methylcholanthrene (3-MC). Both BaP and 3-MC are found environmentally, whereas certain methylated PAHs, such as DMBA, have been synthesized as

model compounds. Benzo(*a*)pyrene and DMBA have been consistently shown in mammalian systems to suppress a variety of immune functional activities, as well as host resistance against microbial pathogens and tumors (White et al., 1994). Indeed, the hallmark immunotoxic effect of most carcinogenic PAHs is a suppressed AFC response. Several mechanisms of PAH immunotoxicity have been hypothesized, including membrane perturbation, altered interleukin production, and disruption of $[Ca^{2+}]_i$ mobilization (White et al., 1994); however, the mechanism receiving most attention concerns AhR binding, subsequent activation of the Ah gene complex, and the CYP1 family of P450 monooxygenases (Silkworth et al., 1984; Willett et al., 2001).

Benzo(a)pyrene

Despite the common occurrence of BaP in the aquatic environment (BaP and other PAHs have been detected in contaminated sediments throughout North America at levels as high as 21,200 μg PAH per g) (Collier et al., 1985), its suggested association with clinical pathologies in feral fish populations, and its well-established immunosuppressive and carcinogenic effects in mammalian models, little is known regarding the effects of BaP on the immune response of fish. Holladay et al. (1998) demonstrated reduced phagocyte respiratory burst activity and splenic/pronephric lymphocyte counts (compared to controls) in BaP-exposed tilapia (*Oreochromis niloticus*). Altered phagocyte activity was also reported following BaP exposure of rainbow trout (*Oncorhynchus mykiss*) (Walczak et al., 1987) and sea bass (*Dicentrarchus labrax*) (Lemaire-Gony et al., 1995). Smith et al (1999) demonstrated that i.p. injection of BaP significantly reduced the AFC response of tilapia; however, alterations were found to be highly dependent on the dosing schedule. In the most comprehensive *in vivo* study examining the immunomodulating effects of BaP in fish, Carlson et al. (2002a,b, 2004) demonstrated that a single i.p. exposure to subtoxic doses of BaP (2, 20, or 200 μg/g BW) suppressed immune function and host resistance in Japanese medaka (*Oryzias latipes*). Injection with 2 μg BaP per g BW significantly suppressed mitogen-stimulated T- and B- lymphoproliferation (in the absence of elevated hepatic CYP1A expression/activity) and at the two higher concentrations significantly suppressed AFC numbers, $\cdot O_2^-$ production, and host resistance against challenge with *Yersinia ruckeri*. Moreover, using the low-affinity AhR agonist benzo(*e*)pyrene (BeP) and the AhR antagonist/CYP1A inhibitor α-naphthoflavone (ANF), Carlson et al. (2002b, 2004) also demonstrated in medaka the importance of the AhR pathway and CYP1A-mediated production of reactive BaP metabolites in mediating BaP-induced immunotoxicity. *In vitro* mammalian studies by White et al. (1994), demonstrating the amelioration of BaP-induced immunotoxicity in rodent cells by ANF, support the medaka findings, as do the *in vitro* studies by Faisal and Huggett (1993) demonstrating the amelioration of BaP and BaP-diol-epoxide-induced immunotoxicity by ANF in spot (*Leiostomus xanthurus*).

3-Methylcholanthrene

Although the amount of information concerning the effects of 3-methylcholanthrene (3-MC) on the immune response of fish is extremely limited, studies in mammals have demonstrated its ability to alter both acquired and innate immunity; for example, exposure of sRBC-immunized mice to 3-MC produced a marked depression in serum antibody titers (Davila et al., 1995). In addition, exposure of rodents to 3-MC has been shown to alter T-lymphocyte proliferation, NK cell cytotoxicity, and cytokine production. Little consistent information exists regarding the effects of PAHs on rodent innate immunity, but the nonspecific immune defenses of fish appear to be particularly sensitive to the immunomodulating effects of 3-MC. Studies by Reynaud et al. (2001) have demonstrated that i.p. exposure of carp (*Cyprinus carpio*) to 40 mg 3-MC per kg produces a rapid increase in MØ respiratory burst. Maximal immune alterations coincided with peak induction of cytochrome P450 and ethoxyresorufin-*O*-deethylase (EROD)/glutathione *S*-transferase activity in the liver and head kidney. Exposure of 3-MC-exposed animals to ANF reversed PAH-induced immune alterations, thus suggesting metabolite-mediated activity. In a later study by the same authors (Reynaud et al., 2002), the role of $[Ca^{2+}]_i$ levels in mediating 3-MC-induced ROI production by activated carp MØ was examined *in vitro*. Using AhR antagonists, it was determined that some mechanism other than $[Ca^{2+}]_i$ release was responsible for the PAH-induced upregulation of free radicals.

7,12-Dimethylbenzanthracene

7,12-Dimethylbenzanthracene (DMBA), a synthetic PAH often used as a prototype PAH in controlled laboratory studies, has well-known carcinogenic and immunotoxic properties (Dean et al., 1983). Despite the fact that field studies have linked aquatic PAH contamination with neoplastic changes in inhabiting fish and an association appears to exist between carcinogenesis and immunosuppression, only limited data are available concerning the immunotoxic effects of DMBA in fish. In a study in which tilapia (*Oreochromis niloticus*) were injected for 5 consecutive days with milligram quantities of DMBA (5 or 15 mg DMBA per kg), lymphoid organ cellularity was significantly reduced (Hart et al., 1998); alterations in immune functional activities (i.e., phagocytosis and free-radical production) were only produced at those DMBA concentrations that produced acute toxicity. It was suggested that hematopoietic organ cellularity might be a more sensitive indicator of DMBA exposure than are changes in immune function.

Immune dysfunction has also been reported in feral fish populations inhabiting PAH-contaminated aquatic environments; for example, fish collected from a river heavily contaminated with creosote (Elizabeth River; 5.6 µg BaP per g sediment) had significantly suppressed NCC activity, MØ-mediated chemotaxis, phagocytosis, and respiratory burst activity, and mitogen-stimulated lymphoproliferation (Faisal et al., 1991a,b; Weeks and Warinner, 1984; Weeks et al., 1986, 1988, 1990). Maintenance for several weeks in clean water reversed the aforementioned immunosuppressive effects. Several studies have also demonstrated immune dysfunction following exposure of laboratory-reared fish to PAH-contaminated environmental samples. Rainbow trout (*Oncorhynchus mykiss*) exposed in microcosms to liquid creosote exhibited reduced MØ oxidative burst activity and peripheral B-lymphocyte numbers (Karrow et al., 1999). Moreover, injection of Chinook salmon (*Oncorhynchus tshawytscha*) with a PAH-contaminated sediment extract (10 µg BaP per kg sediment) decreased host resistance against infection with *Vibriosis anguillarum*; salmon injected with a laboratory-prepared PAH mixture (6.3 µg total PAH per g BW containing 0.378 µg BaP per g BW) also had suppressed resistance against infection (Arkoosh et al., 2001). Finally, winter flounder (*Pleuronectes americanus*) exposed to a PAH-containing crude oil sediment for 4 months demonstrated significantly lower numbers of hepatic MA compared to flounder raised on clean sediment (Payne and Fancey, 1989).

Future Directions

The historic animal models for investigating chemical-induced immunotoxicity are rodents, particularly mice (rats are commonly used for more routine toxicological research); however, rising social and political concern regarding the use of mammalian species for scientific studies has given rise to the search for alternative species. Because of their versatility and immune system similarities with mammals (including humans), teleost species appear to be a logical choice as an alternate model for immunotoxicological investigations. One such species is the Japanese medaka (*Oryzias latipes*). In the summer of 1994, the space shuttle *Columbia* lifted-off for a 2-week mission into outer space. On board were four Japanese medaka. Their mission, which they successfully completed, was to become the first vertebrates to successfully reproduce in space (Ijiri, 1995). This event was just one "first" in the long history of medaka biological research that spans nearly a century (Wittbrodt et al., 2002). These tiny fish with big eyes, indigenous to Japan, Korea, Formosa, and China, have been widely used for studies in modern genetics (particularly to study nervous system and ocular development), transgenics (Muir and Howard, 1999), classical toxicology, and carcinogenesis.

Medaka provide a number of advantages over other fish or mammalian species for scientific research. These advantages include their small size (<1 g BW); transparent embryos, which allow identification of abnormalities during embryonic development; short generation time; easy breeding under laboratory conditions; adaptability to a wide range of water temperatures and salinity; low cost to maintain; sensitivity to chemical-induced carcinogenesis; low incidence of spontaneous tumors; and easily identifiable sexual characteristics. On the other hand, immunological reagents such as cytokines, cell lines, and monoclonal antibodies readily available for rodent species are more difficult to obtain for fish, in general.

Medaka have been used in this and other laboratories to investigate the effects (and underlying mechanisms) of aquatic stressors on immune function and host resistance (Beaman et al., 1999; Carlson et al., 2002a,b, 2004; Duffy et al., 2002, 2003; Zelikoff, 1998; Zelikoff et al., 1996, 2000, 2002). In this regard, a battery of immune assays (originally developed for use in rodents) (Luster et al., 1988) has been validated in medaka for assessing xenobiotic-induced effects on immune function and host resistance. The potential immunotoxicity of several classes of chemical contaminants (metals, pesticides, insecticides, PAHs, and HAHs), complex mixtures (groundwater), and physical stressors (hypoxia and temperature stress) has been investigated using this model. Thus, taken together, medaka appear to be an appropriate model for demonstrating the immunotoxic potential of commonly occurring aquatic stressors. In addition, immune assays adapted for use in this species also appear to serve successfully as early indicators of the effects of chemically polluted aquatic environments.

Conclusions

The immune system is exquisitely sensitive for assessing the toxic effects of chemicals and physical stresses of environmental concern. Adverse effects on the immune system are, for the most part, observed before more routine markers of chemical stress and toxicity, such as enzyme induction, tissue cellularity, and hematological changes (Dean et al., 1983; Luster et al., 1988). The sophistication and complexity of the immune system enable it to (potentially) be the most sensitive and therefore most prominent body function to detect harmful effects from environmental stressors. The sensitivity of this system seems to reside in the complex interactions that must occur for the mobilization of immune defenses. Disruption of any of these processes could offset the balance necessary for immunoregulation in the host and thus produce a cascade of detrimental secondary events, including compromised host resistance against infectious diseases and cancer.

Because of the sensitivity of the immune response to environmental toxicants and its importance for maintaining host resistance against disease, chemical-induced immune dysfunction can be predictive of the toxicological hazards and risks associated with pollutant exposure. The previous establishment of highly sensitive immune assays to enumerate these alterations has culminated in a well-characterized battery of endpoints that can be used successfully to predict biological impact and adverse health outcomes in exposed populations. Such sensitivities also have major applications in efficacy-testing programs including those following remediation. Information on host immunocompetence generated from such programs could aid in management decisions regarding the effectiveness of any remedial activities and the rates of recovery of affected sites. It could be assumed that changes in directions indicating decreased exposure and effects of affected sites precede an improvement in the ecological health of the environment; thus, assays that measure immune dysfunction can serve as rapid indicators of the direction of change in the toxic exposure and effects at a particular monitoring site.

It is important to recognize that, although alterations in host immunocompetence and immune function are measured in a singe individual, these effects often result in decreased resistance to infectious agents and cancer and thus affect the population at large. Conclusions from field and laboratory studies suggest that chemical contaminants weaken hosts' immune defenses, making them more susceptible to epizootic infections. Measures sensitive enough—and a monitoring system broad enough—to identify immunologic and environmental trouble in time to head off major catastrophes are needed.

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12

Chemical Carcinogenesis in Fishes

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CONTENTS

Introduction

Among the industrialized nations of the world, the potential for causing human cancers has been a great concern in risk assessments of environmental pollutants for many years. In contrast, this concern has historically occupied a lower level of interest among those evaluating the risk of chemical hazards to wildlife, including fishes. This is because ecological risk assessments on animals are primarily concerned with higher order effects, such as impacts on populations and communities. This is a sharp contrast to human assessments, where for obvious reasons the individual is of paramount concern. It is noteworthy that for both fish and humans, cancer is not generally considered a disease that has large impacts on population and community variables, such as reproductive success, population densities, community diversity, and so forth.

Nevertheless, chemical carcinogenesis in fishes has received considerable attention among aquatic toxicologists, and their studies have contributed much to our knowledge base in recent years. There are several motivations for this line of research. Numerous epizootics of cancers in free-living fishes have been observed, and the evidence for a chemical etiology is oftentimes much stronger than that available for human cancers. Thus, in addition to generating concern for the health of specific populations of fishes, these epizootics have presented themselves as useful "laboratories" for exploring chemically mediated carcinogenesis in real-world, complex scenarios that are relevant to human exposures. Additionally, fish provide excellent models for the study of chemical carcinogenesis with relevance to both fish and human health. Several small fish species models have been developed for these studies that allow for screens for carcinogens that are very cost and time effective relative to standard *in vivo* rodent exposures. Studies of biochemical and molecular mechanisms of carcinogen metabolism, DNA damage and repair, and oncogene activation in these and other, generally larger fish species have revealed that many features of these

processes are qualitatively shared among fish and mammalian models, though important, oftentimes species-specific differences have been observed as well. The rapid advancement of molecular biology is accelerating the elucidation of these similarities and differences, and their underlying basis. Relatedly, fish comprise by far the most diverse class of vertebrates and thus provide a vast resource for studies in comparative biology (Powers, 1989); for example, studies of related species of fish that display marked differences in susceptibility to chemically induced cancer may help identify critical phenomena underlying carcinogen sensitivity in specific groups of organisms, including fish and humans. Conversely, the shared evolutionary history of fish and humans has resulted in opportunities to study process fundamental to all species (e.g., signal transduction) in organisms biochemically less complex than humans.

Herein, we provide a historical perspective on this subject and describe the state of the science of chemical carcinogenesis in fishes, including discussions of specific classes of carcinogens, carcinogen metabolism, and molecular and biochemical mechanisms of mutagenesis, tumorigenesis, and DNA repair. We also describe current laboratory and field approaches for investigating carcinogenesis in fishes. Portions of these discussions will be placed in a comparative context with mammalian models, for which more information is oftentimes available. For detailed discussions of chemical carcinogenesis in mammals, the reader is referred to Pitot and Dragan (1991).

Historical Perspective

As a result of the intensification and acceleration of laboratory and field investigations of neoplasia in fish during the past three decades, the body of knowledge in this highly specialized facet of cancer research has been much expanded in breadth and depth. The refined laboratory toolkit was deployed improving epidemiology of neoplasia in fish and supporting the association between environmental contamination and this form of chronic toxicity. In the laboratory, molecular biology coupled with refined and directed biochemical studies have uncovered events and their modulation that enhance or diminish tumor development. In the analysis of the pathogenesis of the disease, new methods to more critically evaluate the role of specific lesions were deployed. In retrospect, one can recapitulate events as follows.

Early Discoveries

Prior to mid-20th century, neoplasms had been known to occur in fishes but information about them was limited to a few anecdotal descriptive reports (Wellings, 1969). Among the first reports was a liver cell tumor diagnosed as an adenoma in a blue shark (*Prionace glauca*) in 1908 (Schroeders). This anecdotal era ended abruptly in the mid-1950s and early 1960s, when in Italy, France, and the United States large epizootics of hepatic neoplasms began to be recognized at many hatcheries, almost exclusively in rainbow trout (*Oncorhynchus mykiss*, then *Salmo gairdneri*). By 1965 it was established that aflatoxins in pelleted hatchery foods were the cause of the liver tumors (Halver and Mitchell, 1967; see review by Sinnhuber et al., 1977). These epizootics of liver neoplasms became the first instance in which the occurrence of fish tumors led to the recognition of a new family of chemical carcinogens. The epizootics also led to recognition that the rainbow trout is more sensitive to the carcinogenic effects of aflatoxins than are other salmonids and most, if not all, mammals that have been tested to date.

Until 1963, salmonids were the only family of bony fishes in which liver tumors had been found, and all significant epizootics of fish with liver tumors had come from hatcheries. Perhaps because it was known that salmonids would develop tumors after exposure to appropriate compounds, this opened the door for workers using the trout model to investigate factors that modulate carcinogenesis, a theme that not only continues to be actively pursued but is also of fundamental importance in risk assessment.

As we will see, those investigating other species of fishes took different approaches, either sampling many individuals from a particular field site or, as in the case of small aquarium fishes, using proven mammalian carcinogens in laboratory exposures followed by serial bioassay to determine alterations. The latter led to a more in-depth appraisal of the pathogenesis of neoplasms in liver and in other organs. In 1963, Dawe et al. (1964) performed a survey of feral fishes from Deep Creek Lake, Maryland, and

found cholangiocellular neoplasms in 3 of 12 white suckers (*Catostomus commersoni*) and one hepatocellular neoplasm among 100 brown bullheads (*Ictalurus nebulosus*). This small set of findings accomplished three things: (1) it showed that the salmonid family is not the only family of teleosts in which liver tumors can arise, (2) it triggered the suspicion that anthropogenic carcinogens in aqueous habitats might be responsible for liver neoplasms in feral fishes, and (3) it raised the question as to whether hepatic and other neoplasms in fishes might serve as indicators of the presence of chemical carcinogens in geographically widespread aqueous habitats. Within 2 years of the Deep Creek Lake survey, Stanton (1965, 1966), using a small fish of the cyprinid (minnow) family, *Brachydanio rerio* (or "zebra danio"), demonstrated that diethylnitrosamine (DEN) and cycasin are hepatocarcinogenic in that species.

These three discoveries, all made in rapid succession within a 5-year period (1961 to 1966) were largely to determine the pathways taken by investigations of neoplasms in fish up to the present time. The main directions these investigations took can be categorized as follows:

1. Rainbow trout have been evaluated experimentally, as they are a highly sensitive animal for the detection and bioassay of the hepatocarcinogenic action of chemicals and are an apparently ideal species for analysis of the histo- and cytogenesis of liver tumors. More recent investigations have proven this to be a multi-organ carcinogenesis model.
2. Surveys of polluted marine and freshwater habitats have been conducted with the purpose of finding epizootics of neoplasms in fishes as evidence of environmental exposure to carcinogens (for reviews, see Couch and Harshbarger, 1985; Harshbarger and Clark, 1990).
3. Many different species of small "aquarium fishes" have been tested to determine their sensitivity to a wide variety of known chemical carcinogens and their future usefulness in bioassays of chemicals for carcinogenic potency (reviewed in Boorman et al., 1997; Bunton, 1996; Hawkins et al., 1995; Hoover, 1984). Work with these and other aquarium species underwent rapid expansion. As a consequence of such successful employment of fishes in carcinogen testing, an investigation of the underpinning molecular etiology of cancer has been facilitated by three aquaria-held species. *Xiphophorus*, medaka, and zebrafish have each emerged as models of particular human cancers (for a general review, see Berghmans et al., 2005; Ostrander and Rotchell, 2005; Stern and Zon, 2003).

Association of Fish Cancers with Environmental Contaminants

The Dawe et al. (1964) study is one of the earliest if not the first report that suggested an association between anthropogenic carcinogens in the aquatic medium and neoplasia in fishes. Epizootics of neoplasia in wild fishes have been reported following carefully conducted, often multi-year, investigations in the United States (Harshbarger and Clark, 1990; Malins et al., 1985), Canada (Goyette et al., 1988), various countries bordering the North Sea (Vethaak and Rheinallt, 1992), and Japan (Kimura et al., 1984). The approach used in searching for epizootics in wild fishes has been to establish the prevalence of neoplasia in organisms from specific sites and to compare this with that of organisms from reference sites. Because cancer is a form of chronic toxicity, there has been an association between advanced age of fish and the prevalence of tumors (Baumann et al., 1990). In addition, those fish residing on or near sediment (benthic species) have often been those in which tumors have been found (Murchelano and Wolke, 1985; Myers et al., 1994; Peters et al., 1987; Vethaak and Rheinallt, 1992). In general, the characteristics of the tumor-prone fish include multiple years of age, residence in polluted harbors or estuaries, and a bottom- or benthic-dwelling lifestyle (Harshbarger and Clark, 1990).

A more thorough coverage of field studies and fish neoplasia is provided later in this chapter; however, an overview of approaches and findings to establish the association between environmental contamination and neoplasia is presented here and organized around principles presented by Peters et al. (1987) and reviewed and expanded by Vethaak and Rheinallt (1992). According to these principles: *"A correlation must exist between disease* (*neoplasia*) *prevalence and distribution of a pollutant within a relatively small area."* Studies by Malins and associates (1985, 1987) meet these conditions. English sole (*Parophrys vetulus*) were collected from Eagle Harbor and from a reference site in Puget Sound,

Washington. Hepatocellular and cholangiocellular carcinomas were found in 27% of 75 fish examined. Sediments from the contaminated region contained particularly high concentrations of aromatic hydrocarbons and a variety of nitrogen-containing aromatic compounds. Much lower concentrations of aromatic hydrocarbons were found in the reference site and fish collected there were free of neoplasms.

"There are parallel tendencies for changes in disease prevalence and changes in pollution level over a long period of time" and *"A reduction in the level of pollution results in a decline in the prevalence of the disease"* are two closely related requirements. Both were met in analyses by Baumann and Harshbarger (1995). After a coal coking facility was closed in 1983, polycyclic aromatic hydrocarbons (PAHs) in sediment and in brown bullhead catfish tissues showed marked decline when measured in 1987 (Baumann and Harshbarger, 1995). Over the same time span, in the 3- to 4-year old resident catfish, liver cancer prevalence declined to about one quarter of the 1982 frequency.

"The disease occurs regularly in heavily polluted waters but is seldom if ever encountered in [clean] waters." Many field investigations have met this criterion (Malins et al., 1985, 1987; Pinkney et al., 2001; Vogelbein et al., 1990). The study by Pinkney et al. (2001) was particularly relevant as the epizootic was not only of liver neoplasia but pancreatic neoplasms as well. This finding had never before been reported. *"Field data are confirmed by long-term experiments."* Extracts of contaminated sediment from sites where resident wild fish showed elevated tumor prevalence produced tumors of the same type in exposed laboratory surrogate fish (Fabacher et al., 1991). *"Disease prevalence is related to body burdens of pollutants."* The extensive field investigations from Alaska to southern California reported in Myers et al. (1994) demonstrated this relationship. This was accomplished directly by establishing body burdens for some contaminants and indirectly by quantifying fluorescence absorbing compounds in bile for others (e.g., PAHs). Pinkney et al. (2001) provide an excellent example of this in their study of reference and contaminated sites in the tidal Potomac River. According to Peters et al. (1987), the probability that pollution is a cause of disease increases when two or more of these criteria are met. From the above, we can conclude that there is a strong, site-specific association between environmental contamination and liver cancer (neoplasia) in fishes.

Multistage Carcinogenesis

Carcinogenesis was believed to arise by a multistage process involving initiation, promotion, and progression (Figure 12.1). This has been a long-standing paradigm that is now shifting, yet much of the fish-related literature is still based on it. More recent literature in the medical arena shies away from the traditional paradigm and refers instead to agents as either genotoxic or nongenotoxic and are discussed later. It was in rodent experimental systems that this stepwise process was first demonstrated and used to investigate numerous compounds. It is also important to remember that the initiation–promotion model was first described in skin and subsequently substantiated in liver, colon, lung, prostate, and mammary gland of rodents. For our considerations in this chapter on carcinogenesis in fishes, only skin, liver, and perhaps colon apply; in the case of colon, fish may lack a distal portion of the gut that is strictly analogous. Given the lack of demonstration of initiation and promotion in fish skin neoplastic development, we need only consider liver. As will be shown below, liver carcinogenesis in trout and medaka has included initiation and promotion. Progression has received little attention overall, but at least one report (Okihiro and Hinton, 1999) established the occurrence of this stage in medaka.

As mentioned previously, the initiation–promotion–progression hypothesis is somewhat dated. Other early general rules, such as "All carcinogens are mutagens" and "All mutagens are initiators," are also now known to be false. There remain compounds and conditions that alter gene expression without causing mutations and may promote (by either increasing tumor yield or reducing the time between exposure and appearance of tumor). Also, from studies that addressed the proportion of known carcinogens that were detected by Ames assay (about 60%), it is apparent that there are nongenotoxic carcinogens. Some, therefore, prefer to reject the old paradigm and instead discuss carcinogens more broadly as either genotoxic or nongenotoxic. Given that the majority of publications relating to fish are based on this paradigm, however, we shall discuss this topic within that original framework.

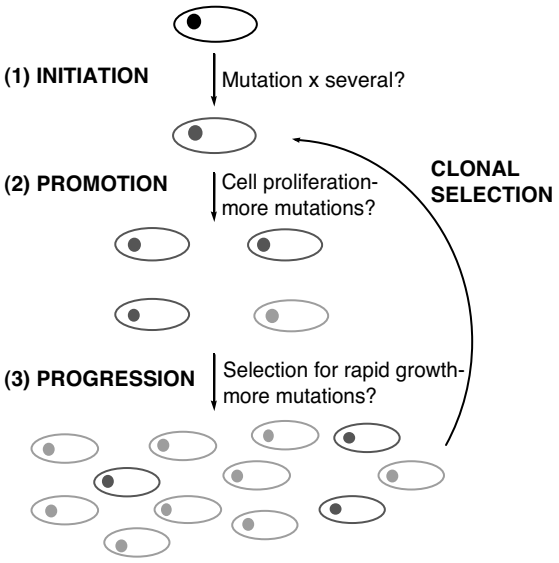


FIGURE 12.1 The multistage process of carcinogenesis.

Initiation is an irreversible mutation in the DNA of a somatic cell. The Millers (reviewed in Miller and Miller, 1983) gave us the appreciation for the chemicals that could initiate carcinogenesis and for the process itself. The chemicals were electrophiles or were metabolized (general case) to an electrophilic state. Electrophiles then bound to nucleophilic centers in DNA, forming DNA adducts. If the DNA was replicated prior to repair or repaired wrongly and the cell's DNA underwent replication prior to repair, the mutation was "fixed." As will be presented in subsequent sections, carcinogen exposure regimes in trout and medaka often involve larval and juvenile stages. These stages are characterized by rapid growth, and it is likely that some of the sensitivity to the compounds arises from the relatively rapid cell division in growing tissues. The morphologic indicator of initiation is the formation of foci of cellular alteration, and determining the number of foci is one way to measure the initiation potential of a given test agent (Pitot and Dragan, 1991).

Promotion, in the traditional paradigm, is brought about by compounds that may not, by themselves, be carcinogenic. They typically do not bind to DNA; rather, they allow for the clonal expansion of initiated cells by providing a selective growth advantage. In the context of liver carcinogenesis, the initiated cells (focal clones) show preferential growth when compared to nonfocal tissue nearby. Determining the growth of foci is a measure of promoting capacity of the promoter or condition. This is now known to involve such factors, in certain conditions, as inhibition of protein phosphatases causing the cell to remain in a hyperphosphorylated state favoring proliferation. In promotion, the so-called reversible stage of neoplasia, cell proliferation is required for clonal expansion of initiated cells. Some promoters are thus carcinogenic yet nongenotoxic, involving epigenetic mechanisms (those that affect gene expression rather than gene structure) and inducing cancer in cells that have already mutated. There are, however, also examples of compounds that are complete carcinogens that are classed as promoters. These highlight the advantage of genotoxic vs. nongenotoxic, rather than promoter, classification.

Progression is the least well characterized of the stages. This is thought to involve accumulation of further genetic alterations in a population of initiated cells that have been provided a growth advantage through promotion. Tumor cell heterogeneity may serve as evidence of progression; for example, almost the entire tumor is a clone with a given phenotype, but with time certain cells of the tumor develop genotypic and phenotypic diversity. It is widely believed that this cellular heterogeneity results from genetic instability acquired during tumor progression. If genetic instability is real, these cells are mutated at rates in excess of those of the surrounding tissue producing subclones. Some of these would be expected to have adaptations that give them a selective advantage. They could escape the host's immune defense system or obtain increased invasive capacity, thus enabling the tumor to extend, move, or migrate

into areas where their selective growth can be further enabled. Okihiro and Hinton (1999) studied progression of hepatic neoplasia in medaka following aqueous exposure to DEN. Larvae (2 weeks of age) were exposed to 350 or 500 ppm for 48 hours and sexually mature adults (3 to 6 months old) were exposed to 50 ppm DEN for 5 weeks. After exposure, fish were maintained in clean water as long as possible to determine the malignant potential and fate of resultant neoplasms. Among 423 medaka examined, a total of 106 neoplasms were included in the complete histological analysis. Metastasis was observed in 19 neoplasms. In addition, those tumors with the highest metastatic potential were hepatocellular and mixed biliary and hepatocellular neoplasms. Neoplastic progression seemed to follow divergent pathways depending on the age and duration of exposure. Brief 2-day exposure was associated with slow progression of foci of cellular alteration to adenomas and then malignant carcinomas. Exposure over longer duration led to brief latency period with rapid development of malignant carcinomas, many of which were invasive and metastatic.

Recent developments have looked broadly at the comparison of tumor progression gene expression profiles and, in a more focused approach, at the role of microsatellite instability status in fish compared with humans. Comparative analysis of microarray data from zebrafish liver tumors and those from selected human tumors revealed considerable molecular conservation at the progression level between fish and humans (Lam et al., 2006). The role of microsatellite instability in the progression of melanomas in *Xiphophorus* has also recently been studied (Zunker et al., 2006). Such instability is involved in human tumor progression, yet its role in fish and whether it is an early or late step is not known; however, these preliminary studies using *Xiphophorus* suggest that this mechanism of instability is not an essential step in melanoma progression.

Most of the work to date has centered on initiation and on detection and enumeration of tumors after varying periods of latency or "grow out." The metabolism of procarcinogens to ultimate carcinogenic form has been a central focus of much of the investigations in trout. DNA adducts have been measured as a means of establishing molecular dosimetry. Some attention has been given to repair, and generally, for the compounds studied, the repair systems of trout are not as robust as those of their mammalian counterparts. Some work has been completed on promoters, and this is reviewed in the subsequent section on modulation of carcinogenesis.

It would be of interest to know which of the fish skin papillomas could be promoted and progressed to malignant neoplasms. Also, we need to know more about those environmental agents that are capable of promotion of papillomas. It should be noted that for tumors that have been reported at other sites in fishes (e.g., swim bladder, forestomach, eye, and thymus) we lack sufficient information to determine whether these fit the initiation–promotion–progression scheme.

Epigenesis relates to the aspects of the environment immediately surrounding the genes (of an altered initiated population of cells) that repress or turn on gene expression. Epigenetic events include those where early changes are induced by carcinogens in target cells and are often tied closely with the cell cycle, either cell proliferation or programmed cell death, or both. The literature on epigenesis in fish, although sparse, is discussed later in this chapter.

In summary, the lack of information for fishes in areas such as epigenesis, in particular, means that coverage of fish carcinogenesis and reviews of the literature now seem somewhat dated. We now know from human studies that genetic and epigenetic events of hepatocarcinogenesis, for instance, are still relatively poorly understood (Herath et al., 2006). Hepatocellular carcinoma (HCC) cells display large genomic alterations, including chromosomal instability, CpG island methylation, DNA rearrangements associated with virus DNA integration, DNA hypomethylation, and microsatellite instability (Herath et al., 2006). Understanding the role of such mechanisms in fish HCC, and other states, is required before the timing of events in malignant transformation can be determined.

Classes of Chemical Carcinogens Investigated in Fishes

There are many classes of chemical carcinogens, some for which effects in fish have been studied and others that are regarded as emerging classes whose carcinogenic effects have yet to be investigated in depth. Here we discuss in turn aflatoxins, polycyclic aromatic hydrocarbons, alkylating agents, and

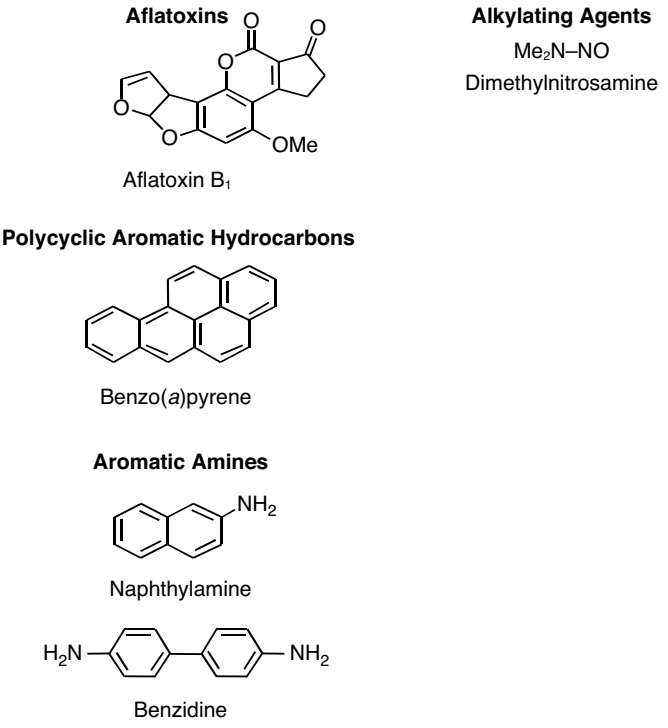


FIGURE 12.2 Common classes of environmental contaminants that act as initiating agents.

aromatic amines (Figure 12.2), for each of which examples concerning development of cancers in fish have been reported. A short discussion of potential new classes of chemical carcinogens then follows.

Aflatoxins are fungal toxins produced by species such as *Aspergillus flavus* that grow on grain and peanut crops in humid conditions. Aflatoxins are a major contributory cause of human liver cancer in the tropics, mainly as a result of ingesting contaminated foods. Fish, particularly trout, have been experimentally exposed to these compounds in their role as a surrogate for humans in examining the toxicological effects (Bailey et al., 1998; Tilton et al., 2005). Trout are selected due to their well-documented sensitivity to aflatoxin B_1 (AFB_1), and channel catfish are often selected as a comparatively resistant species. Fish used in aquaculture, including tilapia and channel catfish, have also been the subject of aflatoxin studies, primarily to investigate the effect of contaminated foodstuff (Lim et al., 2001; Manning et al., 2005).

Polycyclic aromatic hydrocarbons (PAHs) are the pyrolysis products of fossil fuels such as oil, coal, tobacco smoke, and grilled meat. Benzo(*a*)pyrene (BaP) is frequently used as a model carcinogen, although the most potent carcinogenic hydrocarbons in mammalian models are actually 7,12-dimethyl-benzanthracene (DMBA) and dibenzo(*a*,*l*)pyrene (DBP). All are involved in the genesis of major cancers in mammals but are not in themselves carcinogenic; they act instead as promoting agents after biotransformation. Laboratory studies have begun to investigate the causal relationship between a number of PAHs and neoplasia in a number of fish species. Research has involved tank-held fish and exposures to defined chemicals, including BaP, DMBA, DBP, and chemical mixes of various PAHs, to investigate the role and mechanism of PAH-induced cancer in fish (Rotchell et al., 2001a). Such studies aim to address progression in terms of histopathology but also describe specific molecular-level changes occurring during tumor development.

Aromatic amines are used in the dye and rubber industries. Examples include β-/α-naphthylamine, now banned because of their role in human bladder cancer, and dimethylaminobenzene, a food dye used to color margarine which is also banned because of its role in causing liver and bladder cancers in humans. Nitrosamines are a third class; these are procarcinogens that require enzymatic activation to

create an ultimate carcinogenic compound. Such aromatic amines are detectable in the aquatic environment, and a study of their biological effects, including tumor induction, in fish has been conducted (Linjinsky, 1993).

The classes of potential carcinogens include nanomaterials and estrogens. These are currently not strictly chemical carcinogens, however, because their accepted mechanism of action is considered physical oxidative damage of DNA, although this perception may change. Nanomaterials are currently used in many biotechnology applications. Recent studies using rodents have shown that fullerenes can bind to DNA, distorting its structure and stability (Zhao et al., 2005), but little else is known about their toxicity in any organism. A single study to date has shown that uncoated fullerenes can cause oxidative damage in fish, potentially leading to DNA damage (Oberdorster, 2004). It is thus too early to determine whether such chemicals are genotoxic or carcinogenic in fish.

Estrogens are metabolized by various enzymes, to several metabolites including catechol estrogens. These, in turn, can lead to quinone production, free-radical production and, again, oxidative damage. As has been reported for fullerenes, there is also new evidence of estrogen metabolite DNA adducts in vertebrate species (Bolton et al., 2004; Roy and Singh, 2004), although in fish this has yet to be examined.

Three major classes of common environmental contaminants have been listed—aflatoxins, PAHs, and aromatic amines—and all are considered initiating agents with the common feature that they all possess electrophilic (electron-deficient) groups. Two potential emerging classes have been suggested, although less is known about their potential mode of interaction with biological macromolecules. The biological macromolecule, DNA, is extremely nucleophilic and as such is highly susceptible to attack by electrophilic compounds, especially during replication when the DNA helix is unwound from its chromatin packaging. In addition to their initiating effect, recent studies suggest that several of these chemical carcinogen compounds also elicit nongenotoxic effects that lead to the development of cancer.

Carcinogen Metabolism

Most carcinogens are believed to induce cancer by directly or indirectly altering DNA, especially the DNA associated with the synthesis or regulation of protooncogenes and tumor suppressor genes, as discussed later in this chapter. Chemicals that induce cancer in animals can be classified as those that are direct acting or those that require metabolism (bioactivation) to reactive intermediates. Bioactivation of most precarcinogens to active carcinogens involves the cytochrome P450 (CYP) system, which has been discussed in detail in Chapters 4 and 5. The sections of this chapter dealing with carcinogen metabolism, alteration of DNA, and DNA repair are especially interrelated, as the type of DNA alterations induced by carcinogens is determined by the metabolic pathway and metabolites produced, and the ability of an organism to repair DNA damage is influenced in part by the type of DNA alteration. This section focuses on four chemicals: AFB_1, DMBA, 2-acetylaminofluorene (AAF), and BaP. These specific chemicals are well-characterized carcinogens in mammalian systems. In fish these four chemicals range from highly potent carcinogens to very weak carcinogens. Rainbow trout are exceptionally sensitive to AFB_1-induced carcinogenesis, DMBA is carcinogenic in most fish, various teleost species exhibit a range of sensitivities to BaP-induced cancer, and most fish are resistant to AAF-induced carcinogenesis. Examining these compounds is particularly useful in highlighting both similarities and differences in metabolism, DNA damage, and DNA repair between fish and mammals.

AFB_1

Aflatoxin B_1 is a mycotoxin that is known to be hepatocarcinogenic in mammals, including humans (IARC, 1993). Different species exhibit different sensitivities to AFB_1-induced carcinogenesis. Among rodents, rats are relatively sensitive to AFB_1, whereas mice and guinea pigs are relatively resistant (Degen and Neumann, 1981; Raj et al., 1984). Similar species differences in AFB_1 sensitivity are observed in fish; salmon, catfish, and adult zebrafish are resistant to AFB_1, whereas the Shasta strain of rainbow trout is more sensitive to AFB_1-induced tumors (Bailey et al., 1996; Bauer et al 1972; Hendricks, 1994;

Wales, 1970). The high sensitivity of trout to AFB_1, relative to rodents, is illustrated by the findings that a diet of 4 ppb AFB_1 induced ~60% tumors in trout livers (Sinnhuber et al., 1974), while a 5-ppb AFB_1 diet fed to rats for a comparable time period elicited less than 1/10 the incidence of tumors (Wogan et al., 1974).

Aflatoxin B_1 is metabolized by the CYP system to various metabolites, including hydroxylation to AFM_1 and AFB_1-8,9-epoxide. The epoxide is thought to lead to carcinogenesis due to its high reactivity with DNA (Eaton and Gallagher, 1994; Essigmann et al., 1982, Swenson et al., 1977). Various human CYPs (1A1, 1A2, 2A6, 2B6, 3A4, and 3A5) are capable of metabolizing AFB_1 to the 8,9-epoxide; however, mammalian CYP1A2 and CYP3A4 appear to be especially involved in AFB_1 activation to the carcinogenic epoxide (Gallagher et al., 1996; Hengstler et al., 1999). In trout, CYP2K1 appears to be primarily involved in forming the AFB_1-epoxide (Williams and Buhler, 1983; Yang et al., 2000). Differences in both the formation of AFB_1-epoxide and in detoxification reactions appear to contribute to the different sensitivities various species exhibit to AFB_1-induced carcinogenesis. The reactive AFB_1-epoxide can be detoxified by conjugation with glutathione via glutathione *S*-transferases in mammalians. In rodents, the lower sensitivity of mice to AFB_1-induced carcinogenesis, relative to rats, appears to be largely attributed to more efficient glutathione conjugation of the epoxide in mice than in rats (Degen and Neumann, 1981; Raney et al., 1992). In rodents, a high level of glutathione conjugation is associated with a low level of AFB_1–DNA binding (Raj et al., 1984). In fishes, the major excreted AFB_1 metabolites are AFL and AFL–glucuronide conjugates, while glutathione conjugates represent minor metabolites (Dashwood et al., 1992; Gallagher and Eaton, 1995; Toledo et al., 1987; Troxel et al., 1997a). The high sensitivity of trout to AFB_1-induced carcinogenesis is thought to be at least partially attributed to high bioactivation to AFB_1-epoxide by CYP2K1 and to low glutathione conjugation of this reactive metabolite (Dashwood et al., 1992).

DMBA

7,12-Dimethylbenzanthracene is one of the most carcinogenic PAHs in mammals. In fish, DMBA is also a potent carcinogen that induces tumors in liver, stomach, and swim bladder (Aoki et al., 1993; Harrtig et al., 1996; Weimer et al., 2000). Although DMBA is not considered an environmental pollutant, it is modeled after polyaromatic hydrocarbon environmental contaminants. In mammalian cells, DMBA can be bioactivated by cytochrome P450s to a reactive "bay region" diol-epoxide metabolite that subsequently adducts to adenine and guanine residues in DNA (Baird and Dipple, 1977; Diamond et al., 1972; Dipple et al., 1983). The bay region diol-epoxide DMBA-3,4-diol-1,2-oxide is thought to be the ultimate carcinogenic metabolite (Vigny et al., 1985) in mammalian tissues. In addition, DMBA can be activated by one-electron oxidation, as demonstrated by the depurinating DNA adducts observed in mouse skin (Devanesan et al., 1993). Phase I DMBA metabolites can be conjugated via phase II reactions (detoxification) to water-soluble glucuronide, sulfate, and glutathione metabolites.

In rainbow trout, DMBA was shown (Schnitz and O'Connor, 1992; Schnitz et al., 1987) to be metabolized to water-soluble compounds. Initially, most of the identified phase II conjugates were sulfates, especially with low doses of DMBA. As the concentration of DMBA increased, there was a shift to more glucuronide conjugates (Schnitz et al., 1993), consistent with the idea that sulfation has a high affinity with a low capacity, whereas glucuronidation has a low affinity with a high capacity (Pang et al., 1981). In cultured primary trout liver cells, DMBA was also rapidly converted to water-soluble metabolites (Weimer et al., 2000). Enzymatic digestion indicated that ≤5% of these metabolites were conjugated with sulfate, 10 to 35% were glucuronide conjugates, and the majority of these metabolites remained unidentified. Depleting cellular glutathione did not significantly alter the metabolism of DMBA to water-soluble metabolites or the proportion of sulfate or glutathione conjugates. Schnitz et al. (1993) also reported a large fraction of DMBA metabolites that were not identified. Although the rate of metabolism of DMBA to water-soluble metabolites was similar between mouse embryo fibroblasts and cultured trout liver cells, the level of unconjugated, nonpolar (phase I) metabolites was much lower in trout liver cells than in mammalian fibroblasts (Weimer et al., 2000). This finding suggests that phase II metabolism (detoxification) of DMBA primary (phase I) metabolites is more efficient in trout liver cells than in mammalian fibroblasts.

Various mammalian CYPs have been implicated in the formation of the genotoxic bay region diol epoxide. The PAH-inducible isozymes of the 1A and 1B families, as well as the phenobarbital-inducible isozymes of the 2B family, have been shown to participate in mammalian metabolism of DMBA (Diamond et al., 1972; Dipple et al., 1984; McCord et al., 1988; Morrison et al., 1991). Jefcoate's laboratory (Christou et al., 1994; Pottenger and Jefcoate, 1990; Savas et al., 1993) identified CYP1B1 as a prominent enzyme metabolizing DMBA to the 3,4-diol in mammalian cells. A CYP1B1 ortholog has been identified in teleosts (Godard et al., 1999a,b); however, the capacity of this enzyme to metabolize DMBA has not yet been reported. Although recent studies have examined the role of fish CYPs in DMBA metabolism, it is not yet clear that DMBA 3,4-diol is the proximate carcinogen or DNA-reactive metabolite in these animals (see below). Liver microsomes from β-naphthoflavone (BNF)-treated trout produce several DMBA metabolites, predominantly an unidentified polar metabolite, 2-OH-DMBA, and 4-OH-DMBA, with lesser amounts of the DMBA 8,9-, 5,6-, and 3,4-dihydrodiols (Miranda et al., 1998). A purified CYP1A1, in the presence of added epoxide hydrolase, produced a similar spectrum, with DMBA-8,9-dihydrodiol being the major metabolite (40% of total) and the 3,4-dihydrodiol being less than 3% of total. Furthermore, in trout liver cells (Weimer et al., 2000) the major DMBA metabolites identified were 10,11-, 8,9-, and 5,6-DMBA dihydrodiols and DMBA 2-, 3-, or 4-phenol. 7-OH-Methyl-12-methyl-benz(*a*)anthracene and 12-OH-methyl-7-methyl-benz(*a*)anthracene were minor metabolites, and only a very small amount of DMBA-3,4-dihydrodiol was detected. Polar metabolites that did not migrate with any DMBA metabolite standards were also observed. In addition, Weimer et al. (2000) demonstrated that pretreating cultured trout liver cells with BNF to induce CYP1A1 or with α-naphthoflavone (ANF) to inhibit CYP1A1 activity resulted in significant increases and decreases, respectively, in conversion of DMBA to water-soluble metabolites. Although these studies may imply a role of CYP1A1 in general DMBA metabolism, other studies indicate it is not possible to extrapolate the effects of known inhibitors of mammalian CYP450s to trout (Miranda et al., 1997). Furthermore, modulating cultured trout liver cell CYP1A1 activity with BNF or ANF did not significantly influence the level of DNA adducts, indicating that other CYPs may be responsible for metabolizing DMBA to DNA-reactive metabolites.

BaP

Benzo(*a*)pyrene is an environmental PAH pollutant, and different species of fish exhibit different sensitivities to BaP-induced cancer. Liver is a common target of BaP-induced carcinogenicity, and CYP1A appears to be primarily responsible for converting BaP to the 7,8-dihydrodiol and the ultimate carcinogen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene (BPDE) (Kelly et al., 1993a,b; Williams and Buhler, 1984). Glucuronide and glutathione conjugates of BaP are formed in different fish cells. Although >90% BaP-7,8-diol was recovered as glucuronic acid conjugates from bluegill cells, rainbow trout cells primarily formed glutathione conjugates, and brown bullhead cells formed both types of phase II conjugates, but at a much lower rate (Smolarek et al., 1987). Some fish (killifish, brown bullhead catfish, English sole) residing in PAH-contaminated waters develop a high incidence of liver tumors, while other fish (e.g., oyster toadfish, channel catfish, starry flounder) have a much lower incidence of tumors (Baumann and Harshbarger, 1995; Collier et al., 1993; Harshbarger and Clark, 1990). Using BaP as a model PAH, species differences in phase I and phase II metabolism cannot explain the differential susceptibility of different species to BaP-induced carcinogenesis. Channel catfish (PAH-resistant species) have higher basal and induced CYP1A activities than the more PAH-sensitive brown bullhead catfish (Ploch et al., 1998; Watson and Di Giulio, 1997). Although channel catfish appeared to have a higher phase II capacity than brown bullhead catfish (Hasspieler et al., 1994) these two species did not exhibit different sensitivities to oxidative DNA damage, assessed as 8-hydroxy-2-deoxyguanosine levels (Ploch, 1997; Ploch et al., 1999). Furthermore, *in vitro* ^{3}H-BaP–DNA binding levels were much higher using microsomes prepared from channel catfish than from brown bullhead micosomes (Hasspieler et al., 1994), consistent with higher CYP1A activities in channel catfish. A higher level of BaP–DNA adducts, however, was formed *in vivo* in brown bullhead catfish than in channel catfish (Hasspieler et al., 1994), suggesting that factors in addition to phase I and phase II activities are involved in BaP-induced DNA adduct formation and carcinogenicity in different catfish species. Furthermore, studies by Willett et al. (2001) indicated that *in vivo* brown bullhead preferentially formed BaP-7,8-dihydrodiol, a finding consistent

with the higher sensitivity of this species to BaP-induced hepatocarcinogenesis and DNA adduct formation *in vivo*. These observations may indicate that an enzyme different from CYP1A is responsible for bioactivating BaP in fish. In this regard, BaP is more efficiently metabolized to the 7,8-diol by human CYP1B1 than by CYP1A (Shimanda et al., 1999). Although CYP1B1 has been identified in fish (Godard et al., 1999a,b), the possible role of this isozyme in BaP metabolism in catfish is not yet known. Additional studies are required to delineate the mechanistic reasons why brown bullhead and channel catfish, as well as other fish species, display differences in BaP-induced liver cancer.

2-Acetylaminofluorene

2-Acetylaminofluorene (AAF) is a carcinogen in most mammalian systems. AAF has been studied as a model chemical to learn about the metabolism and mechanism of action of carcinogenic aromatic amines and amides (Miller et al., 1961). In mammals, AAF is *N*-hydroxylated to N-OH-AAF, a proximate carcinogenic metabolite (Miller et al., 1961; Weisburger and Weisburger, 1973). AAF can also be deacetylated to 2-aminofluorene (AF), which can also form DNA adducts. On the other hand, ring hydroxylation of AAF by other CYP450s results in the formation of water-soluble conjugates that are excreted (Lotlikar and Hong, 1981). AAF can also be metabolized by flavin monooxygenases (Kitamura et al., 1999). The sensitivity of different species to AAF-induced carcinogenesis is thought to be due in part to the balance between metabolic activation to reactive, DNA-damaging agents and metabolic deactivation/conjugation to water-soluble compounds which are eliminated from the organism (James et al., 1994; King, 1978; Miller et al., 1961). Relative to most mammalian species, fish generally are less sensitive to AAF-induced carcinogenesis, and some species (e.g., rainbow trout and medaka) are more resistant to AAF-induced carcinogenicity than other fish (e.g., guppies) (James et al., 1994; Lee et al., 1968). Exposing trout to AAF orally resulted in rapid elimination of AAF (Steward et al., 1994), and in the bile most AAF metabolites (5-/7-/8-/9-OH-AAF) were conjugated with glucuronides. Only a small fraction of AAF was identified as N-OH-AAF (the glucuronide conjugate). Similar results were observed in isolated trout liver cells treated with AAF (Steward et al., 1995) and in trout (Elmarakby et al., 1995) and medaka (James et al., 1994) liver microsomes incubated with AAF. Because mammalian CYP1A2 evolved from relatively recent gene duplication, and a true CYP1A2 ortholog is not present in trout (Morrison et al., 1995), these animals may be deficient in the ability to *N*-hydroxylate AAF, which likely plays a role in resistance to AAF-induced carcinogenesis. In addition, exposing trout liver cells to N-OH-AAF did not result in the formation of detectable DNA adducts (M. Miller, unpublished observations), indicating efficient detoxification of this metabolite in trout. Furthermore, trout and medaka appear to have relatively low activity of three other enzymes implicated in the metabolic deactivation/conjugation of AAF (*N*-hydroxy-AAF-sulfotransferase, glucuronyltransferase, and *N*-hydroxy-AAF acyltransferase) (Elmarakby et al., 1995; James et al., 1994). Deficiencies in AAF-activating enzymes, coupled with a relatively high capacity of fish for ring hydroxylation (detoxification), likely contribute to the resistance of most fish to the hepatocarcinogenic action of AAF.

Carcinogen Alteration of DNA

Chemicals previously classed as initiators and promoters now tend to be described as either genotoxic or nongenotoxic agents. The former include compounds that are electrophilic and interact directly with any of the four nucleotide bases within the DNA structure, forming adducts. The latter exert their influence via epigenetic mechanisms and are discussed later.

Specific Characterized Adducts

AFB_1

Among fish and mammals, AFB_1 mainly forms adducts with guanine (Figure 12.3), and 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–AFB_1 is the major adduct formed. Over time, this adduct forms open-ring N^7-formamidopyrimidine adducts (Bailey et al., 1994; Croy et al., 1980). AFL also appears to form the

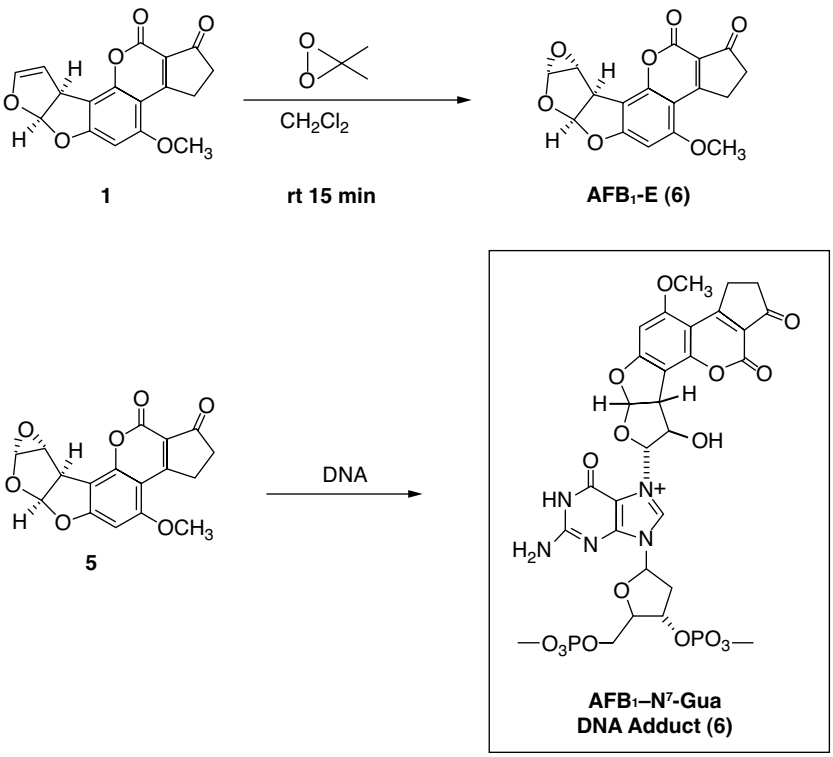


FIGURE 12.3 Metabolic activation of AFB_1 and DNA adduct formation.

same guanine adduct as the epoxide metabolite in trout (Bailey et al., 1994). In trout, AFB_1–DNA adducts are associated with G→T transversions in codons 12 and 13 of Ki-*ras* (Bailey et al., 1988); however, in rats, although the same G in codon 12 of *ras* is targeted in AFB_1-induced liver tumors, the prominent mutation observed is a G→A transition, with minor G→T transversions in the same codon. Although AFB_1 targets the same nucleotide in trout and rat *ras*, it is puzzling that presumably the same type of AFB_1–deoxyguanine adduct in *ras* codon 12 results in different types of prominent mutations between trout and rats. A thorough comparison of reactivity of mammalian and trout DNA replication polymerases may reveal differences in the way the polymerases interact with AFB_1–DNA adducts.

DMBA

In mammalian embryo cells, three prominent DNA adducts are derived from DMBA diol-epoxide: *anti* dihydrodiol epoxide-deoxyguanosine, *anti* dihydrodiol epoxide-deoxyadenosine, and *syn* dihydrodiol epoxide-deoxyadenosine (Pigott and Dipple, 1988). Various mutations attributed to these adducts have been described. In the mammary gland of Big Blue rats, DMBA induced mutations in lacI, and A→T (44%) and G→T (25%) transversions were the predominant types (Manjanatha et al., 2000). In addition, DMBA can be activated by one-electron oxidation, as demonstrated by the depurinating DNA adducts observed in mouse skin (Devanesan et al., 1993). DMBA is known to bind covalently to DNA in trout, and there is evidence that the overall level of adduct formation is comparable to that in mammals (Schnitz and O'Connor, 1992). In trout, however, a much different spectrum of adducts in liver Ki-*ras* was demonstrated (Fong et al., 1993), with G→T transversions and G→A transitions dominating in codon 12. Because in mammalian systems the *syn* dihydrodiol epoxide of DMBA nearly exclusively forms adenine adducts, it was predicted (Fong et al., 1993) that the abundance of guanine-based mutations in trout Ki-*ras* may reflect different metabolic paths for DMBA bioactivation. Additional studies indicate that different types of DMBA–DNA adducts are formed in trout cells compared to those adducts formed in mammalian cells. Such differences in DNA adducts may contribute to the differences in types of

DMBA-induced mutations seen between trout and mammalian cells noted above. Smolarek et al. (1987) used high-performance liquid chromatography (HPLC) to characterize DMBA–DNA adducts formed in a trout gonadal cell line and reported a profile of adducts very different from those produced in mammalian cells. Specifically, nonpolar DMBA–DNA adducts arising from the bay region diol epoxide appeared to be absent; however, trout gonadal cells *in vivo* are not known targets for DMBA damage, and the relationship of these adducts to carcinogenesis in fish was not clear. Recently, DMBA–DNA adducts formed in trout liver cells *in vivo* and *in vitro* have been shown to be more polar than those formed in mammalian target cells (Weimer et al., 2000), and only minor levels of the polar adducts derived from the bay region diol epoxide were detected. Incubating trout hepatocytes with DMBA-3,4-dihydrodiol, however, produced three prominent, nonpolar adducts indistinguishable from those in mouse embryo cells (Weimer et al., 2000), suggesting that this compound is not a major metabolite formed in trout or that it is efficiently conjugated or metabolized to an unidentified metabolite. Furthermore, modulating CYP1A1 activity with BNF or ANF in trout liver cells did not significantly alter the level of DNA adducts formed, suggesting other CYPs are involved in metabolism of DMBA to DNA-reactive compounds in trout. In addition, pretreating trout with BNF did not affect hepatic CYP1A1 activity, DMBA–DNA adduct formation, or hepatic tumor response, although tumor response in swim bladder and stomach was significantly reduced (Weimer et al., 2000). Collectively, these results indicate that DMBA bioactivation to DNA-binding metabolites in trout liver cells and mouse embryo cells predominantly involve different metabolic pathways to form different DNA-binding intermediates.

BaP

In both fish and mammals, BaP forms adducts with guanine, as well as other bases, following metabolic activation (Figure 12.4). In brown bullhead and blue gill cells, the major BaP–DNA adduct formed is (+)-*anti*-BaP-7,8-diol-9,10 epoxide with deoxyguanosine (Smolarek et al., 1987). The same adduct is formed in rainbow trout cells exposed to BaP (Smolarek et al., 1987) and in trout exposed to 7,8-dihydrobenzo(*a*)pyrene-7,8-diol (Kelly et al., 1993b); however, significant quantities of an unidentified polar BaP–DNA adduct were noted in trout liver cells exposed to BaP (Smolarek et al., 1987).

Oxidative DNA Damage

As described in the previous section, many organic chemicals can be metabolized to reactive intermediates that can covalently bind to DNA and thereby form stable DNA adducts. In addition to this form of DNA damage, reactive oxygen species (ROS) can interact with DNA and produce another form of DNA damage, oxidative DNA damage. Reactive nitrogen species (RNS) can also participate in these and related forms of DNA damage. Mechanisms by which many environmental contaminants can generate ROS in cells are described in Chapter 6. As with stable DNA adducts, oxidative DNA damage can lead to mutations and cancer initiation. Additionally, ROS can perturb many signal transduction pathways and act as tumor promoters. Marnett (2000) has provided an excellent review of oxidative DNA damage, focused on mammalian models, and a comprehensive monograph concerning free radicals that includes detailed information concerning oxidative DNA damage can be found in Halliwell and Gutteridge (1999).

The species of ROS most associated with oxidative DNA damage is the hydroxyl radical (·OH), the most reactive of the ROS (Ward, 1988). Although superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are less reactive and less damaging to DNA, they can play important roles as precursors to ·OH (see Chapter 6). Additionally, H_2O_2 produced at one cellular localization (such as endoplasmic reticula) can diffuse to another (such as the nucleus) and, via metal-catalyzed Fenton chemistry, yield DNA-damaging hydroxyl radicals some distance removed from ROS production; the inherent reactivity of ·OH precludes such direct mobility of this species. Another oxidant with that has similar effects on DNA as ·OH is peroxynitrite (ONO_2^-), formed by reaction between nitric oxide (NO·) and $O_2^{\cdot-}$ (Koppenol et al., 1992). Other species, including singlet oxygen ($^1O_2$), NO·, and products of lipid peroxidation such as malondialdehyde and 4-hydroxynonenal, can also damage DNA (Halliwell and Gutteridge, 1999; Marnett, 2000).

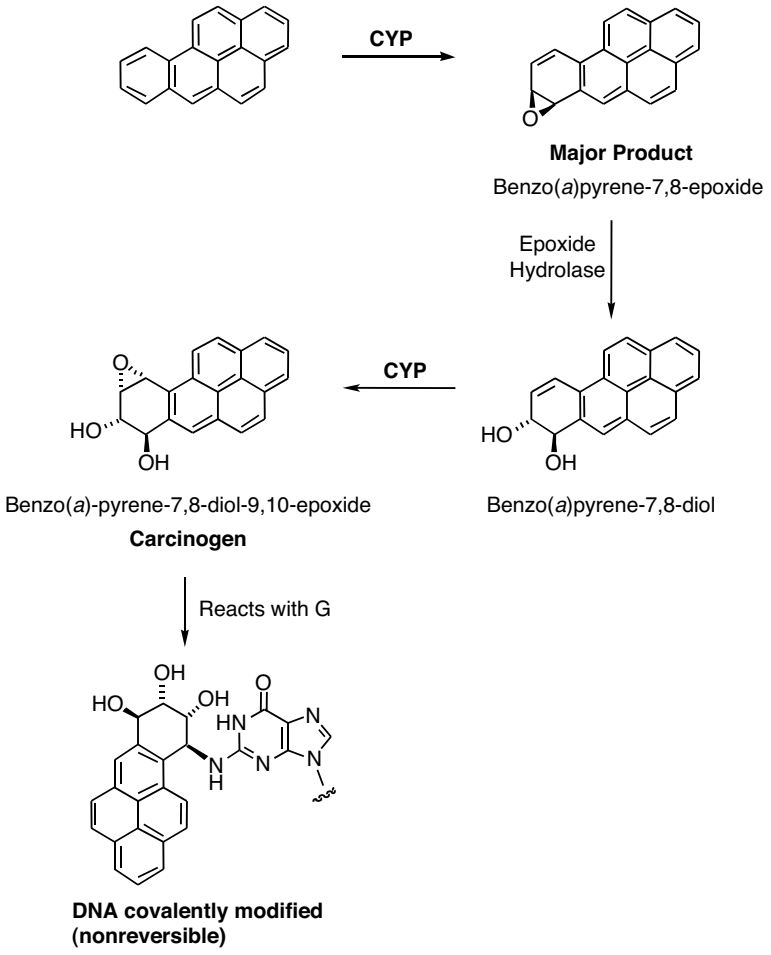


FIGURE 12.4 BaP metabolism and DNA adduct formation.

Direct oxidations of DNA bases, in large part via ·OH, comprise a major form of initial DNA damage by ROS and related oxidants (Halliwell and Gutteridge, 1999) (Figure 12.5). In addition to oxidative reactions, the interaction of $ONOO^-$ with DNA results in the nitration of guanine to form 8-nitroguanine (Figure 12.5). Oxidations occur normally in aerobic cells, a part of the price paid for the energetic efficiency afforded by using O_2 as an electron acceptor; however, many exogenous factors, including many environmental contaminants, can enhance the flux of ROS and increase rates of DNA oxidation (see Chapter 6). Numerous products of oxidative base damage have been identified; an example is shown in Figure 12.6. In addition to their potential roles in carcinogenesis (discussed below), measurements of these products have been employed as markers for oxidative stress in tissues of animals, including fish; however, one must be careful when examining oxidative DNA data, as artifactual oxidations can occur during these analyses which has led to considerable debate concerning true background levels for various tissues and the most accurate approaches for analysis (Cadet et al., 1997; Marnett, 2000).

The consequences of oxidative DNA damage vary considerably with the identity of the base oxidized, the base sequence surrounding the modified base, and the efficiency of DNA repair systems available (Halliwell and Gutteridge, 1999). As an example, 8-oxo-deoxyguanosine (8-oxo-dG), perhaps the most intensively studies oxidized DNA product, is mutagenic; it produces G→T transversions that are frequently observed in mutated oncogenes and tumor suppressor genes (Hussain and Harris, 1998; Shibutani et al., 1991). Thymine glycol, 8-oxo-adenine, 5-hydroxyuracil, and uracil glycol are also mutagenic

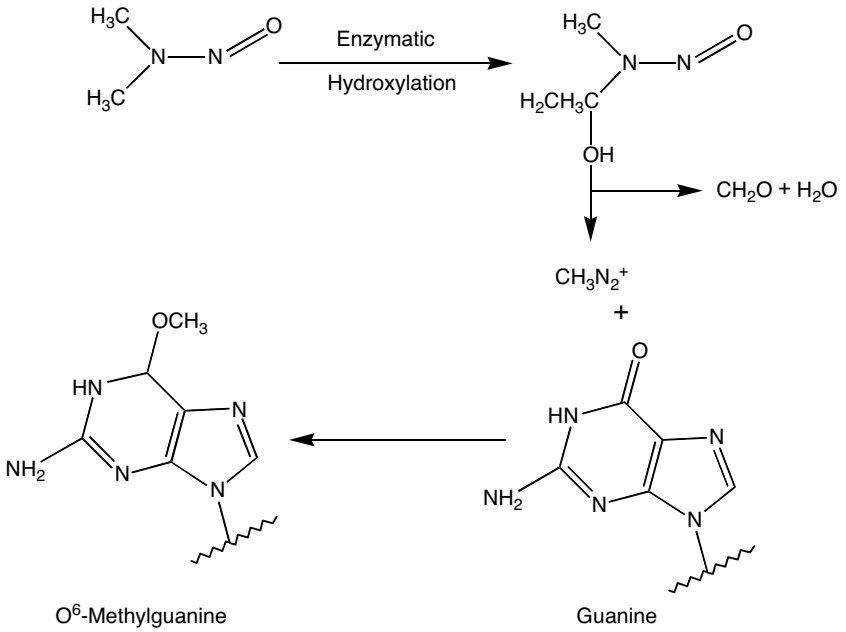


FIGURE 12.5 Nitrosamine-mediated alkylation of DNA bases. Secondary nitrosamines such as *N*-nitrosodimethylamine induce point mutations by alkylation of DNA bases such as guanine to form O^6-methylguanine residues as shown.

(Wang et al., 1998a). Thymine glycol can also block DNA replication and thereby be potentially cytotoxic (Halliwell and Gutteridge, 1999).

Oxidative DNA damage is repaired by DNA repair mechanisms described below; base excision repair is particularly important for the repair of oxidized DNA bases; for example, a FAPy glycosylase removes formamidopyrimidines and 8-oxo-dG, and the human analog of this enzyme is referred to as 8-oxo-dG glycosylase (the OGG1 gene product) (Radicella et al., 1997). The only fish homolog reported to date is for *Tetraodon nigroviridis* (GenBank Accession No. CAG11321). The base-removing action of this and other glycosylase results in abasic sites in DNA, termed apurinic or apyrmidinic sites (AP sites). AP sites, which can occur spontaneously as a result of aerobic metabolism, are themselves mutagenic and are removed by an AP lysase and an AP endonuclease, which cut the DNA on the 3′ and 5′ side of the missing base, respectively. DNA polymerase then fills the resulting one-nucleotide gap with the correct base as guided by the undamaged complimentary strand (Halliwell and Gutteridge, 1999). Nucleotide excision repair, which is generally required for the repair of bases covalently bound to bulky adducts such as PAH metabolites, can also play a role in the repair of oxidative DNA damage. Although the DNA repair machinery is usually very effective in dealing with oxidative DNA damage, it is not completely effective, as evidenced by the presence of oxidized DNA in cells of every aerobic organism examined to date. Increases in the rates of damage above background, by ionizing radiation or ROS-generating chemicals, for example, can increase the levels of damage and associated probabilities of mutations associated with carcinogenesis. Also, this repair machinery itself can be perturbed by ROS and RNS; for example, NO· inhibits several DNA repair enzymes, including FAPy glycosylase, which suggests a potential synergy for NO· to both enhance oxidative DNA damage (via peroxynitrite formation) and inhibit repair of that damage (Laval et al., 1997; Marnett, 2000).

The great bulk of our knowledge concerning mechanisms of oxidative DNA damage and repair is derived from studies of simple model organisms such as bacteria and yeast, and also from mammalian studies; however, it is clear that these phenomena are operative in fish as well, which is certainly not surprising given that fish are aerobic organisms. Nishimoto et al. (1991), for example, reported elevated concentrations of 8-oxo-dG in tissues of the English sole (*Parophrys vetulus*) injected with nitrofurantoin. Increases were most pronounced in liver tissue, with kidney and blood cells affected at the higher

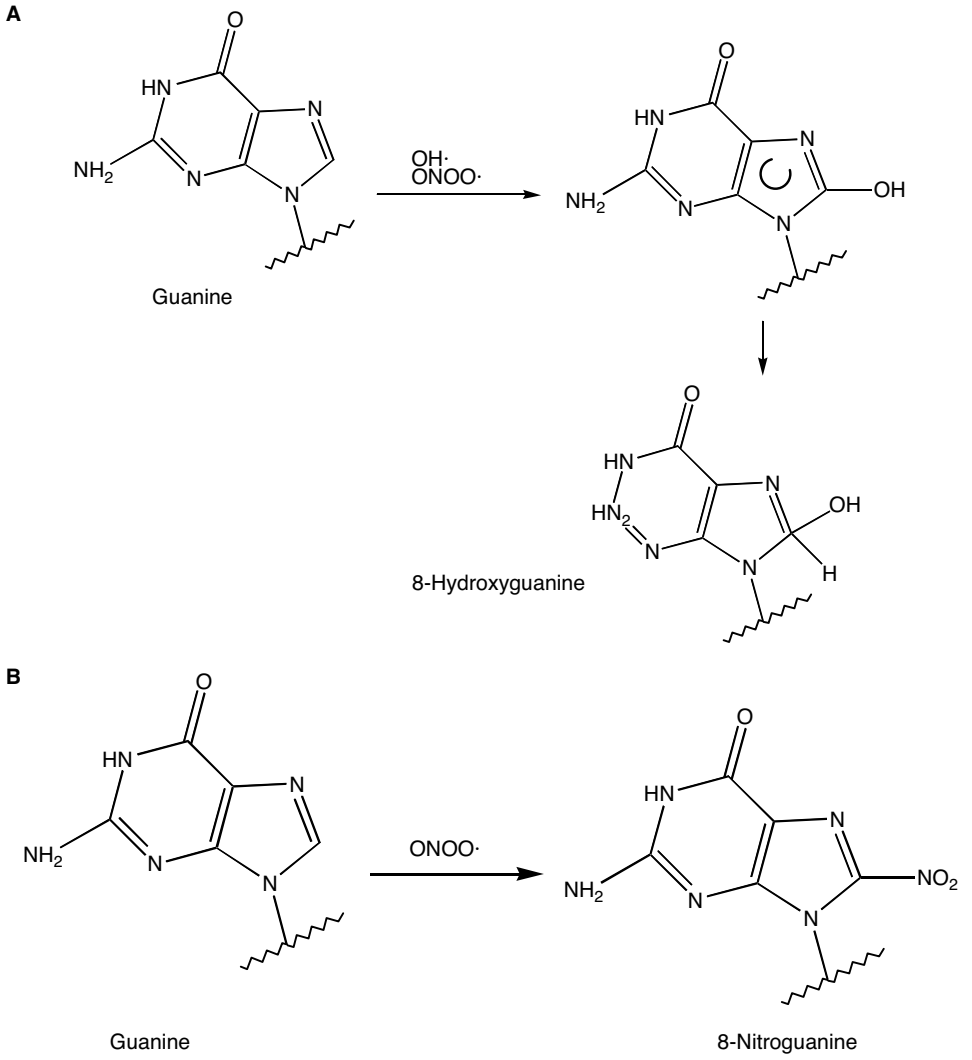


FIGURE 12.6 (A) Hydroxyl radical (OH·)- and peroxynitrite ($ONOO^-$)-mediated oxidation of guanine. (B) Peroxynitrite ($ONOO^-$)-mediated nitration of guanine to form 8-nitroguanine.

exposure concentrations. Likewise, Malins and colleagues (1990) examined DNA from neoplastic livers of feral English sole exposed to carcinogens. Concentrations of the guanine-derived lesion, 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FapyGua), ranged from 0.97 to 5.11 nmol/mg DNA. FapyGua was not detected in non-neoplastic tissue, and this study suggested that reactive oxygen species damage DNA in living systems and thus may play an important role in the formation of neoplastic tissue. In fact, concentrations of 8-oxo-dG in tissues have also been adopted as a biomarker of oxidative stress and environmental pollution in several species of fish including English sole (Malins and Haimanot, 1991), *Sparus aurata* (Rodriguez-Ariza et al., 1999), and *Anguilla anguilla* (Machella et al., 2005).

Chemical–Viral Interactions

Many strains of DNA tumor viruses are known to cause several types of cancer in mammalian models. Also, an estimated 20% of human cancers are thought to have a viral component in their etiology. Human papilloma virus (HPV), for example, can cause intraepithelial neoplasias, or abnormal and precancerous

cell growth, in the vulva and cervix, which can progress to cancer. These tumors often have viral oncogene sequences integrated into the cellular DNA. Examples include the viral E6 and E7 proteins from HPV that bind to and inactivate the *p53* gene and *Rb* genes, respectively, leading to various tumor types (Orjuela et al., 2000); the hepatitis B virus, which alters the *ras* gene and ultimately leads to hepatocellular carcinoma (Kim et al., 2001); and the Epstein–Barr virus, which alters the expression of *p53* and can lead to papilloma formation (Katori et al., 2006). DNA tumor viruses, similarly to certain classes of chemical contaminants, can therefore act as initiating agents of certain cancer types as well as act in the progression of others, although no specific DNA tumor virus action in fish has been investigated to date.

Among fishes, the research effort to date has primarily focused on understanding the retroviral etiologies of various types of cancers and has not addressed the interplay between viral and chemical interactions leading to carcinogenesis. Examples include lymphomas in northern pike (*Esox lucius*) (Sonstegard, 1976), plasmacytoid leukemia in Chinook salmon (*Oncorhynchus tshawytscha*) (Eaton and Kent, 1992), dermal sarcoma in walleye (*Stizostedion vitreum*) (Bowser et al., 1988), probable viral-induced papillomas in brown bullheads (*Ictalurus nebulosus*) and white suckers (*Catostomus commersoni*) (Baumann et al., 1996), viral-induced neurofibromatosis in bicolor damselfish (*Stegastes partitus*) (Rahn et al., 2004), retroviral-induced swim bladder sarcomas in Atlantic salmon (Paul et al., 2006), and retroviral-induced dermal sarcoma in moray eel (*Gymnothorax funebris*) (Buck et al., 2001). Additional studies have strongly suspected, though failed to isolate, a viral infectious agent in the development of fish tumors. Occasionally, a viral etiology is suspected and ruled out using reverse transcriptase activity as an indication of viral involvement. An example is the pigmented subcutaneous spindle cell tumors that affect up to 25% of selected gizzard shad (*Dorosoma cepedianum*) sampled from the Lake of the Arbuckles (Jacobs and Ostrander, 1995; Geter et al., 1998; Ostrander et al., 1995).

Combined, such studies demonstrate that fish are susceptible to retrovirally induced events, although the action of DNA tumor viruses has yet to be investigated. This action may contribute to the multistep progression of the carcinogenesis process in a manner similar to mammalian models. In terms of the mechanism of DNA tumor virus-induced cancers, one might again consider the fish protooncogene and tumor suppressor genes as likely targets. High conservation of the *ras*, *p53*, and *Rb* gene sequences in fish suggests that they may be similarly susceptible to viral activation and inactivation. Indeed, the *Rb* E1A viral binding domain is one of many structurally conserved domains observed in the medaka *Rb* gene (Rotchell et al., 2001a).

Epigenetic Carcinogens

In addition to genotoxic impacts via direct interaction with DNA structure, it is also now well established that multistage chemical carcinogenesis in mammals includes processes under the control of a variety of epigenetic events. Precise mechanisms are still under investigation and have yet to be explored in any depth using fish models. Epigenetic events include those where early changes are induced by carcinogens in target cells and are often tied closely with cell cycle growth: proliferation or programmed cell death, or both (Schneider and Kulesz-Martin, 2004). Several mechanisms have been investigated in mammals. One is the inhibition of apoptosis which helps altered cells escape cell death and adopt a tumorigenic phenotype (Nguyen-Ba and Vasseur, 2006). Tumor suppressor *p53*, for example, is often altered by methylation (as well as by mutation) in certain kinds of human cancers (Agirre et al., 2003). Upregulation of oncoproteins by epigenetic events is another possibility, as is carcinogens binding to and disabling the proofreading enzyme involved in generating new DNA strands (Bignold, 2003). These are all examples of epigenetic mechanisms.

Investigations into epigenetic mechanisms in fish are few. The roles of DNA and histone methylation and transposable element excision have been examined in aquaria-held zebrafish and medaka fish developmental studies (Iida et al., 2006; Rai et al., 2006), although not as yet in any carcinogenesis application. In an environmental application, methylation status in the CYP1A gene promoter of fish sampled from the creosote-contaminated Elizabeth River in Virginia has also been investigated by Timme-Laragy et al. (2005), who postulated that methylation status might account for apparent CYP1A uninducibility but found no such evidence to confirm this. In another study of liver toxicity and carcinogenicity of flounder from the German Wadden Sea coast, investigators failed to find gene mutations and instead

concluded that epigenetic events were the initiating source of the cancers observed (Koehler, 2004), although none was characterized.

One class of nongenotoxic epigenetic carcinogens is selected pesticides. Pesticide examples include chlorothalonol and acetochlor, which promote induction of CYP1A, formation of ROS, and peroxisome proliferation (Freeman and Rayburn, 2006; Rakitsky et al., 2000). Acetochlor is used as a herbicide and is a contaminant found in the aquatic environment at levels of 700 ng/L to highest concentrations of 2.7 μg/L (Helbing et al., 2006). In cell-cycle studies, it was found to significantly reduce the number of cells in the G_1 phase of the cell cycle (Freeman and Rayburn, 2006). Future studies will likely identify many more examples of epigenetic carcinogens and hopefully elucidate their mechanisms of action in fish.

DNA Repair

Overview of Repair Mechanisms

DNA repair is fundamentally important to all cells and organisms. In humans, defects in normal DNA repair are associated with diseases including xeroderma pigmentosum, Cockayne's syndrome, hereditary nonpolyposis colorectal cancer, and Fanconi's anemia; high cancer incidences are commonly associated with defective DNA repair processes (Lehmann, 2003). Different mechanisms or pathways are responsible for repairing different types of DNA damage, and in mammals one type of damage may be repaired by a preferred pathway with high activity, as well as by a different pathway with lower activity for that damage. Such a system operates to ensure the efficient removal of damaged DNA. A brief overview of DNA repair pathways is presented before discussing DNA repair in fishes. Readers are referred to an excellent review of DNA repair for additional information (Sancar et al., 2004).

Direct Reversal (Pyrimidine Dimers)

Direct reversal pathways utilize proteins with a high specificity for a specific type of damage, and the protein directly repairs the damage without synthesizing new DNA. Examples of direct reversal repair proteins are DNA photolyase and O^6-alkylguanine-DNA transferase (ADT). Photolyase recognizes pyrimidine dimers that arise from exposure to ultraviolet (UV) light (adjacent pyrimidine bases in a DNA strand covalently joined following exposure to UV light). Photolyase scans DNA, binds pyrimidine dimers, and utilizes energy from photoreactivating light to break covalent bonds joining bases, thus directly returning the DNA to its original state. ADT in *Escherichia coli* removes methyl groups from the O^6 of guanine and from the O^4 of thymine, and some larger alkyl groups are also removed by this protein. DNA-alkyltransferases function as suicide enzymes; that is, each protein molecule can remove one alkyl group from DNA, the alkyl group becomes attached to the DNA-alkyltransferase, and the protein cannot remove additional alkyl groups.

Deficient/Low Excision Repair (Especially PAH Adducts)

Excision repair is a different type of mechanism for repairing damaged DNA and involves removal of damaged nucleotides followed by synthesis of new DNA. Excision repair can be classified as base excision repair or nucleotide excision repair. In base excision repair, enzymes (DNA glycosylases) recognize damaged bases and initiate the repair process by cleaving the *N*-glycosidic bond connecting damaged base and sugar, creating an apurinic/apyrimidimic (AP) site. AP endonuclease then removes the base-free sugar by cleaving phosphodiester bonds, creating a one-nucleotide gap in the DNA; DNA polymerase replaces the missing nucleotide using the complimentary strand as a template, and DNA ligase forms a phosphodiester bond with the new nucleotide and the original strand, completing the repair process. Different DNA glycosylases recognize different types of DNA damage, including glycosylases specific for uracil, 5-methylcytosine, G–T mispairs, 3-methyladenine, formamidopyrimidine moieties, and pyrimidine dimers. In the case of nucleotide excision repair, a multi-enzyme complex containing endonucleases recognizes damaged DNA, often containing bulky adducts or distorted secondary conformation. Repair is initiated by endonuclease incision on either side of the damaged site,

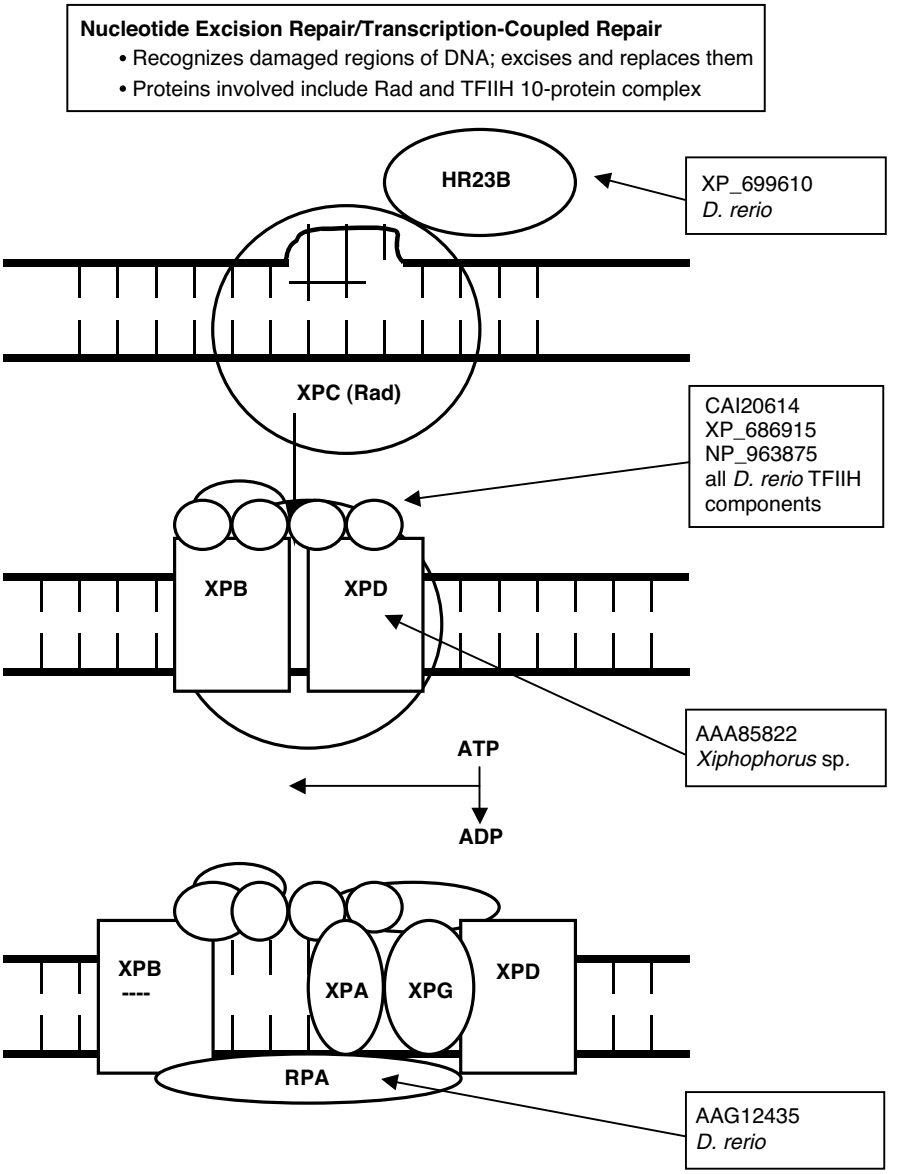


FIGURE 12.7 Overview of DNA repair systems; nomenclature is based on the mammalian systems, and fish homologs, where available via GenBank, are highlighted.

removing a block of nucleotides from the damaged strand. DNA polymerase fills in the resulting gap, and DNA ligase completes the repair process. The presence of multiple repair mechanisms optimizes the probability that DNA damage will be detected and repaired; for example, rather than rely on a single pathway to identify and correct all pyrimidine dimers, in some organisms UV-induced pyrimidine dimers can be repaired by a photolyase, a base excision repair, and by a nucleotide excision mechanism.

DNA Repair in Fish

Little is known about the details of DNA repair mechanisms in fish, relative to what we know of these processes in prokaryotes and mammals (Figure 12.7). A photolyase activity has been described in fish that appears to be the primary mechanism of repairing pyrimidine dimers (Mano et al., 1982; Mitani et

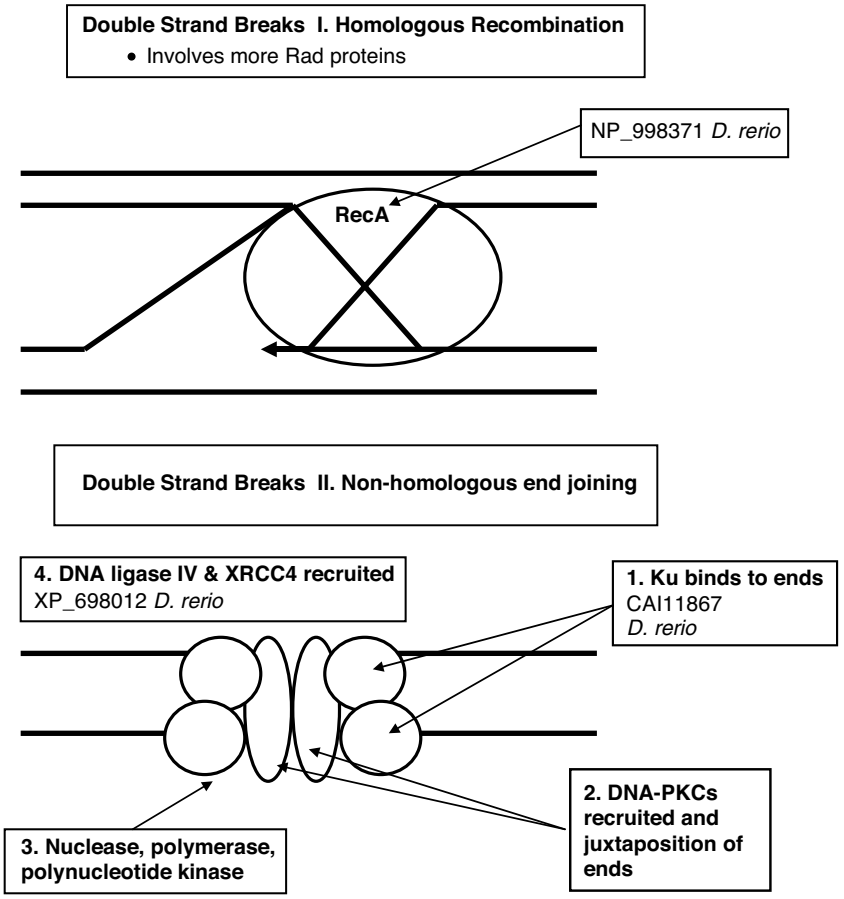


FIGURE 12.7 (cont.)

al., 1996; Nishigaki et al., 1998, Regan et al., 1982; Wales, 1970). Alkyl DNA transferase activity is present in many organisms, and this repair activity has also been detected in fish (Aoki et al., 1993; Nakatsuru et al., 1987). There is also a recent report of aberrant DNA polymerase beta expression in tumor-bearing hybrid *Xiphophorus* species (Heater et al., 2004). A second study observed deficient base excision repair and nucleotide excision repair function in tumor-bearing hybrids (David et al., 2004). These studies combined suggest that DNA repair dysregulation renders fish susceptible to tumorigenesis in a manner similar to mammalian models.

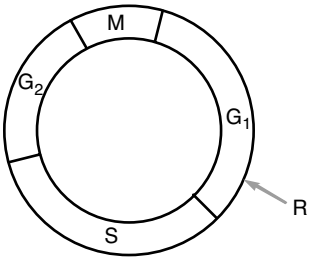
Unscheduled DNA (repair) synthesis (UDS) is an indirect method for detecting DNA excision repair; in the absence of DNA replication, chemical-induced incorporation of [^{3}H]-thymidine into nuclear DNA (assessed by autoradiography or by scintillation counting) is indicative of DNA excision repair. Using UDS, DNA repair has been detected in fish cells; however, the level of UDS induced by carcinogenic alkylating agents is generally lower in trout and other fish cells than in mammalian cells (Ishikawa et al., 1984; Klaunig, 1984; Kelly and Maddock, 1985; Walton et al., 1983, 1984). The level of bleomycin-induced UDS (bleomycin induces DNA strand breaks) in permeabilized trout liver cells is also lower than that in permeabilized mammalian cells, indicating that the decreased sensitivity of trout UDS is not attributed to differences in intracellular deoxynucleotide pools (Miller et al., 1989). Furthermore, the sensitivity of bleomycin-induced UDS to various DNA polymerase inhibitors was similar between trout liver cells and mammalian cells (Miller et al., 1989), suggesting that bleomycin-induced repair proceeds through similar excision repair mechanisms in trout and mammalian cells. Assessing the rate of removing AFB_1– and DMBA–adducts from DNA, a more direct measure of DNA repair, also indicates that the capacity of fish cells to repair alkyl-adducts is lower than observed in mammals.

DNA damage signaling, cell cycle, and apoptosis

The **cell cycle** has distinct phases in growth:

- **M** phase is when mitosis occurs - the nucleus breaks down and the cell divides (cytokinesis).
- **G_1** (First gap) phase is characterized by high biochemical activity.
- **S** (Synthesis) phase is characterized by duplication of DNA and cell contents ready for mitosis.
- **G_2** (Second gap) phase is similar to the first and occurs after duplication of DNA but before mitosis.

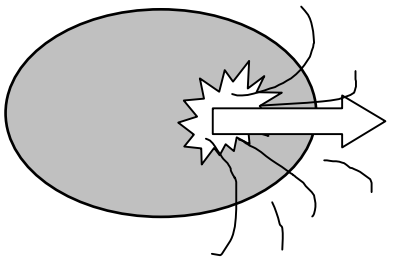
Additionally, there is the **restriction point in G_1**, which is in effect a "point of no return" for the cell.



THE CELL CYCLE

Selected cell-cycle highlights:

- Cell-cycle arrest
  - Chk1, triggers downstream phosphorylation pathways
  - MSH, a homolog of Mut genes
  - Brca1, involved in stalled replication fork restart
- Cell-cycle checkpoint
  - Brca2
  - Rb,
  - AAY41438 *Limanda limanda* (du Corbier et al., 2005)
  - AAG21826 *O. latipes* (Rotchell et al., 2001)
  - XP_695613 *D. rerio*
  - AAS80140 *F. heteroclitus*
- Cell-cycle other
  - Atm, recognizes damaged DNA and phosphorylates downstream proteins such as p53 and Brca1.
  - BAD91491 *D. rerio* (Imamura and Kishi, 2005)



Apoptosis

P53, transcription factor regulated by phosphorylation, stops cell either by holding at a checkpoint or by initiating apoptosis

AAC60146 *O. latipes* (Krause et al., 1997)
CAA70123 *P. flesus* (Cachot et al., 1998)
AAA92052 *X. maculatus*
CAA62450 *Callionymus lyra*
AAY68035 *S. trutta*
AAD34212 *B. barbus* (Bhaskaran et al., 1999)
AAB40617 *D. rerio* (Cheng et al., 1997)
AAC26824 *I. punctatus*

FIGURE 12.7 (cont.)

AFB_1

As discussed above, the same types of AFB_1–DNA adducts are formed in mammalian and fish cells; however, the rate of repair of AFB_1–DNA adducts is quite different between rainbow trout and mammalian systems. The rate of removing AFB_1–DNA adducts in rainbow trout is estimated to be approximately 70 times slower than that in mammals (Bailey et al., 1996). This difference in AFB_1–DNA adduct removal may be a significant factor in the great sensitivity of rainbow trout to AFB_1-induced carcinogenesis.

DMBA and BaP

DMBA– and BaP–DNA adducts have been shown to be repaired at significant rates in mammalian target and nontarget tissue in both *in vitro* and *in vivo* systems (Daniel and Joyce, 1984; Janss et al., 1972; Tay and Russo, 1981). In addition, the susceptibility of different strains of rats to DMBA-induced tumor formation has been found to correlate less with the levels of adducts actually formed than with the length

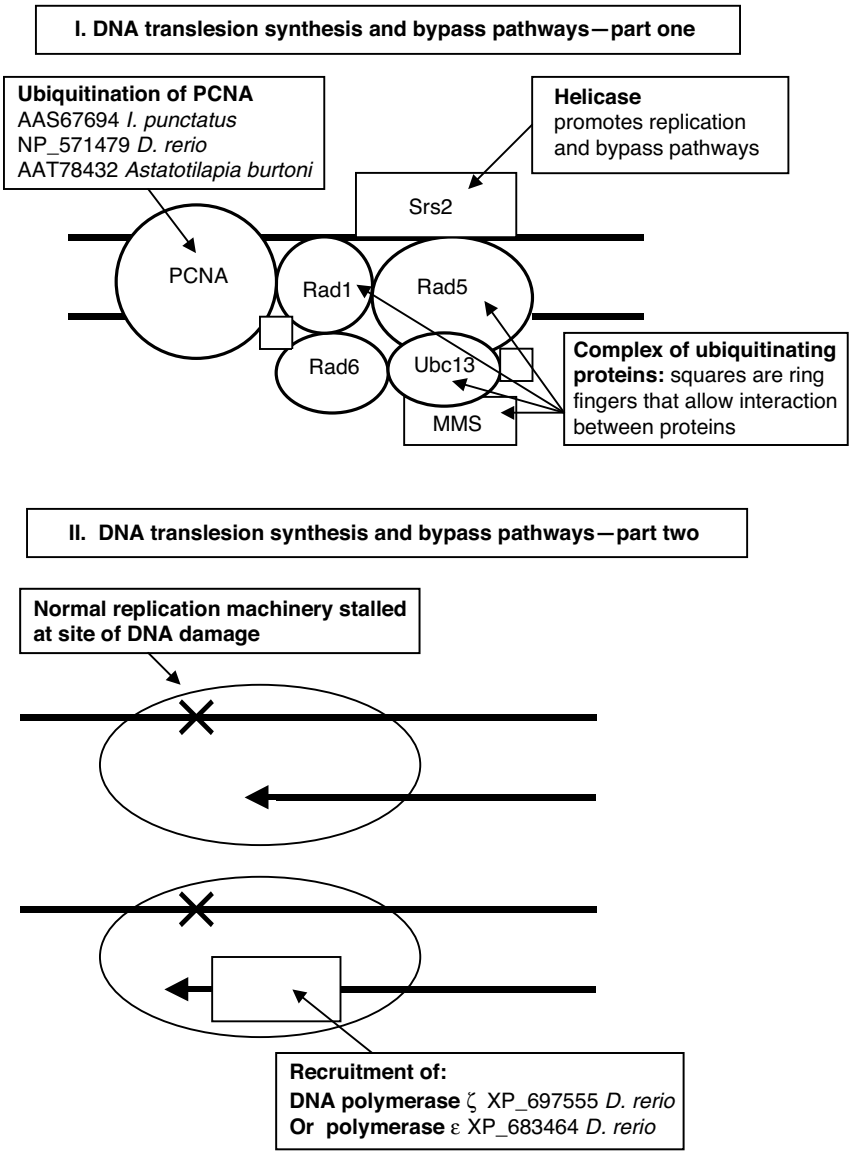


FIGURE 12.7 (cont.)

of time adducts persist (Daniel and Joyce, 1984), indicating the potential importance of DNA repair to DMBA-induced carcinogenesis. Few studies have investigated repair of DMBA– or BaP–DNA adducts in fish. A recent study in cultured trout liver cells (Weimer et al., 2000) demonstrated that neither DMBA–DNA nor BaP–DNA adducts were significantly repaired during a 48-hour period, while significant repair of these adducts was detected in mammalian cells during the same time period. This specific observation is consistent with the idea that fish cells have a reduced ability to repair some types of genomic DNA damage caused by bulky adducts, relative to mammalian cells (Bailey et al., 1988; Walton et al., 1983). It is unclear whether the lack of repair of DMBA–DNA adducts by trout liver cells is due to low global excision repair capacity in the teleost system or to formation of adducts that are intrinsically less repairable than those formed in mammalian cells. For example, supporting data were obtained by Willett and colleagues (2001) when they investigated DNA excision repair of UV-exposed hepatocytes in two related catfish species. Neither *Ictalurus punctatus* nor *Ameiurus nebulosus* exhibited any repair over 72 hours when subjected to an endonuclease-sensitive site assay.

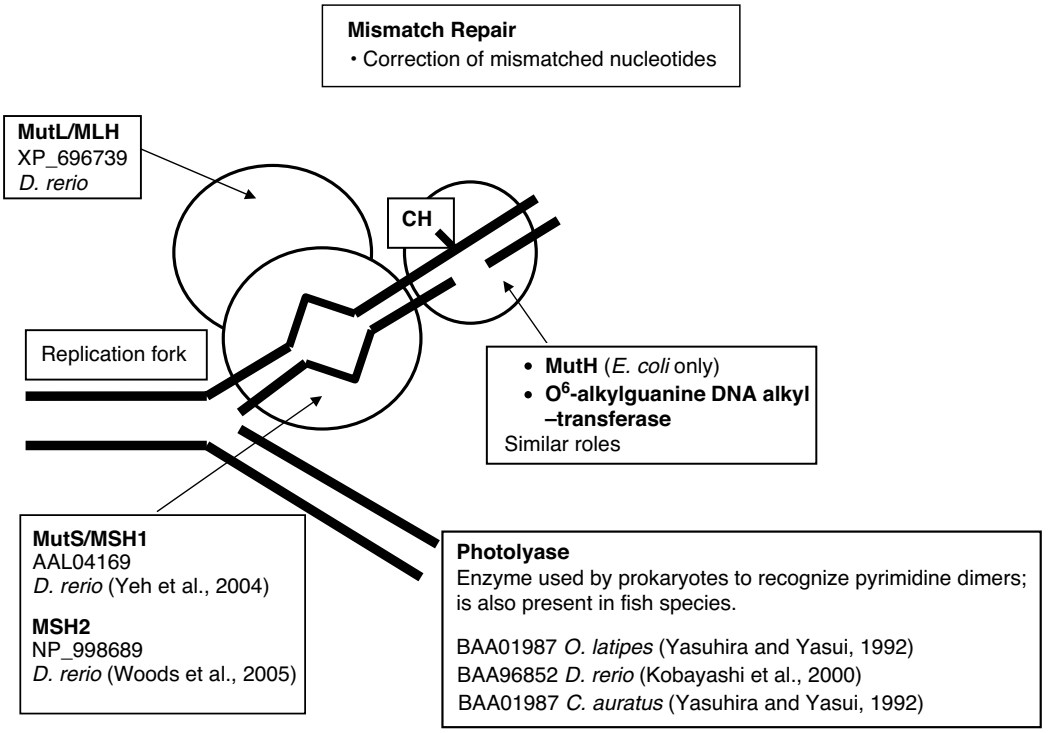


FIGURE 12.7 (cont.)

Molecular Biology of Carcinogenesis

As detailed herein, the primary events responsible for the conversion of a normal cell to a cancerous phenotype occur, in some sense, at the molecular level. Notwithstanding, we focus this discussion of the molecular biology of cancer on the two broad classes of genes that have been implicated in teleost cancer: oncogenes and tumor suppressor genes. Both of these contribute to unregulated cell growth (Figure 12.8).

Oncogenes

Oncogenes were first described in 1976 when the transforming genes of an avian sarcoma virus were found to be present in normal avian DNA (Stehelin et al., 1976). These protein products function as growth factors, growth factor receptors, transcriptional activators, and other components of the signal transduction pathway. In simple terms, they promote cell differentiation and proliferation (i.e., growth). Oncogene activation can occur via point mutations; deletions, insertions, or rearrangement through chromosomal translocation; gene amplification; or proviral insertion and will result in production of protein product phenotype that leads to cancer. Our knowledge of oncogene expression and function in teleost systems lags far behind that of mammals. By 1994, over 60 mammalian oncogenes had been identified and at least a rudimentary understanding of their functions had been described. Conversely, only a handful of oncogenes had been described in fish models (Van Beneden and Ostrander, 1994). Moreover, with the exception of the elegant studies of the *Xiphophorus* sp. model (discussed below), little new information about the function of these genes has been reported from studies of teleost models. For the most part, it appears that oncogene function in fishes is similar to what has been described for mammalian models.

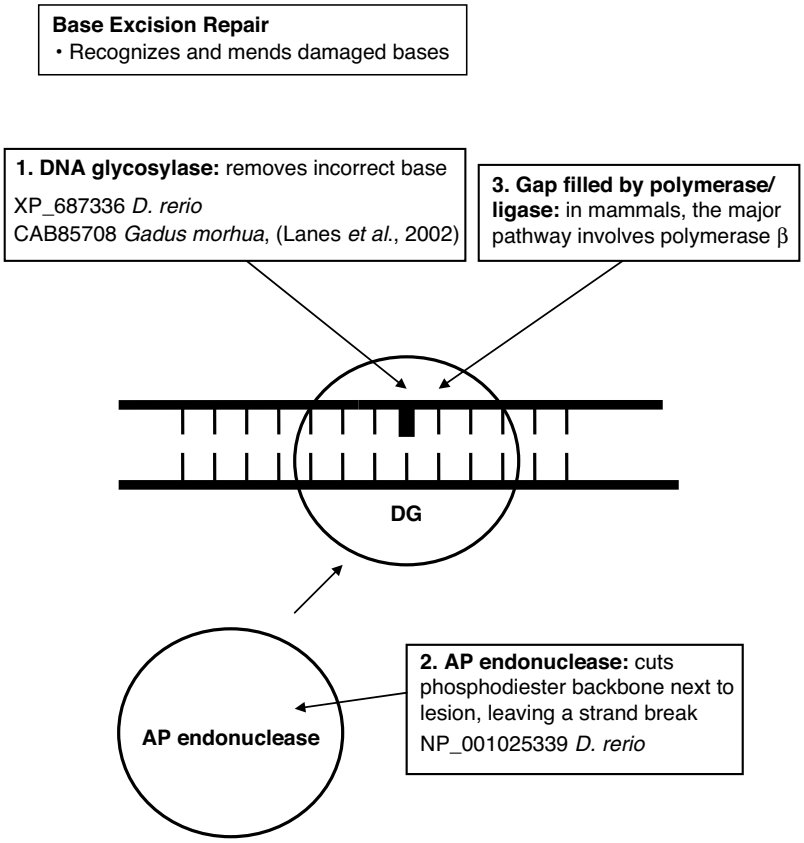


FIGURE 12.7 (cont.)

Classification of Oncogenes

Class I Oncogenes: Receptor and Nonreceptor Protein Tyrosine Kinases

These proteins are membrane bound and catalyze the phosphorylation of tyrosine residues. Tyrosine phosphorylation of intracellular target proteins, as opposed to the more common phosphorylation of serine and threonine residues, often serves as the initial step of signal transduction pathways which control cell proliferation. Among the fishes, receptor tyrosine kinase activity has been best documented in the *Xiphophorus* model system among the *erb*B family (discussed in Schartl and Barnekow, 1982, and below).

The best-studied example of nonreceptor tyrosine kinase activity among fishes is *src*, which has been reported in a variety of primitive fishes, including jawless fish (Schartl and Barnekow, 1982; Suga et al., 1999; Yang et al., 1989), cartilaginous fish (Barnekow and Schartl, 1987; Schartl and Barnekow, 1982), and a variety of evolutionarily advanced fish ranging from catfishes (Yang et al., 1989) to sea robins (Schartl and Barnekow, 1982) to flounders (Barnekow and Schartl, 1987; Schartl and Barnekow, 1982). In addition, a number of partial *src* sequences from other fish species have been deposited with GenBank. The *src* oncogene was the first transforming oncogene to be discovered (Hunter, 1987), and significantly higher *src* tyrosine kinase activity was detected in the malignant melanomas of *Xiphophorus* over that of benign melanomas (Barnekow et al., 1982).

The *src* gene family has at least nine members, including *yes*, *fgr*, *lyn*, *lck*, *fyn*, *hck*, *yrk*, *crk*, and *src*. Of these, *yes* and *fyn* have reported in fish (*Xiphophorus*), and little is known of their function (Hannig et al., 1991). Members of the *abl*-related subgroup of nonreceptor tyrosine kinases are structurally very similar to the *src* family described above. In addition to the *Xiphophorus* model, the c-*abl* gene has been

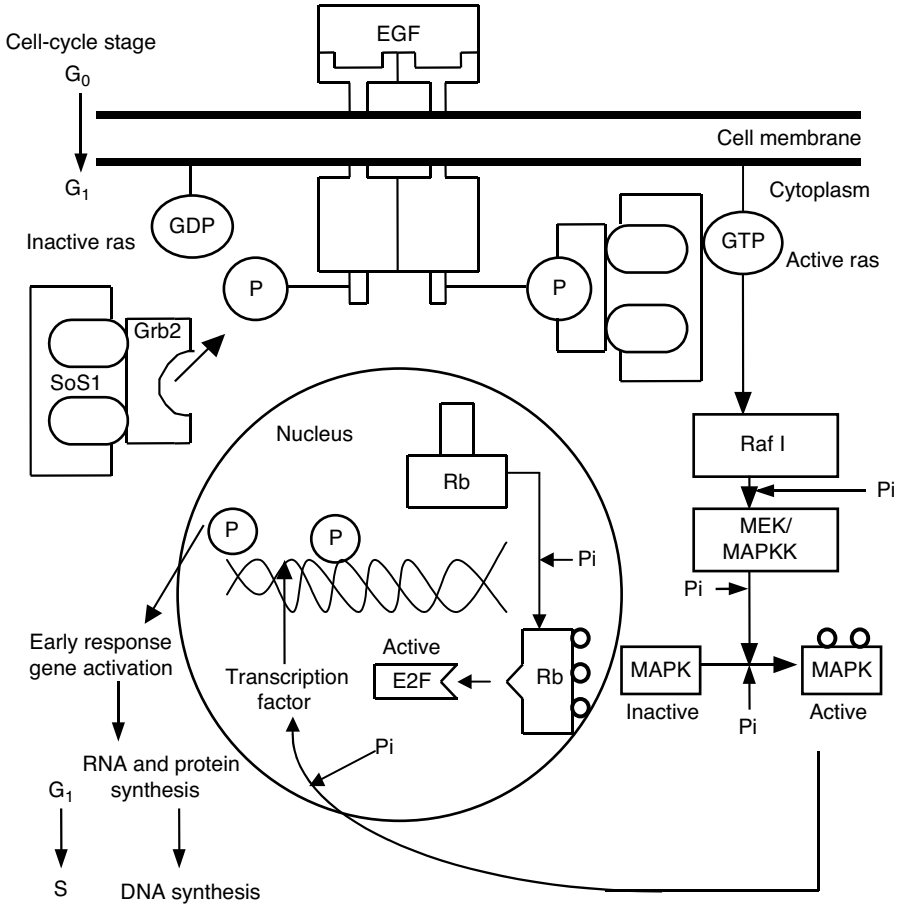


FIGURE 12.8 Overview of the roles of ras protooncogene and Rb tumor suppressor gene in cell signaling. (Ras) An activated receptor tyrosine kinase (EGF in mammals, X*mrk* in fish) binds to an adaptor protein (Grb2) that links to the nucleotide-releasing factor SoS, leading to ras activation and kinase cascade to the nucleus. At the nucleus, activation of early response genes, such as *jun*, *myc*, and *fos*, results in mRNA synthesis and progression of the cell growth cycle to S phase (DNA synthesis). (Rb) Phosphorylation of Rb releases the transcription factor, in this case E_2F. In its unphosphorylated state, Rb inhibits the activity of transcription factors and thus prevents progression of the cell cycle. *Abbreviations:* SoS1, Son of Sevenless (nucleotide exchange factor); Grb2, nucleotide exchange factor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; G_0, resting phase of cell cycle; G_1, gap period in the cell cycle after mitosis and before DNA replication; P, phosphates.

investigated in tomcod (*Microgadus tomcod*) from the Hudson River which exhibit a high incidence of liver tumors (i.e., hepatocellular carcinomas) (Wirgin et al., 1990). Comparison of fish from the Hudson River with a reference population in Maine that does not exhibit tumors revealed significant differences in the allelic frequencies at the c-*abl* C domain. Polymorphisms were not detected at other oncogene loci, including c-Ki-*ras*, c-HA-*ras*, and c-*src*. The authors speculated that tomcod from the Hudson River may be genetically predisposed to the development of hepatocellular carcinomas and, as such, the observed c-*abl* polymorphisms may play a role. Significant further work is necessary before any definite conclusions can be drawn.

Receptor-like protein tyrosine kinases include *ros*, *erb*B, *new*, *fms*, *net*, *trk*, *kit*, *sea*, and *ret*. Among these, *erb*B has received considerable attention, beginning when Downward et al. (1984) reported that the amino acid sequence of six peptides derived from the epidermal growth factor receptor (EGFR) from human placenta were identical at 89% of the residues sequenced, to the transforming protein of the v-*erb*B oncogene of avian erythroblastosis virus. *ret l* has been reported in the zebrafish (Bisgrove

et al., 1997), and several oncogenes with tyrosine kinase activity have been described in *Xiphophorus*, including the melanoma gene (*Tu*/*x-egfr*B/X*mrk*/*x-erb*B*) and a related *erb*B gene (*x-egfr*A) (discussed below).

Class II Oncogenes: Growth Factors

It appears that some oncogenes function as growth factors or exhibit growth-factor-like activity. The best-studied example is probably the simian sarcoma virus oncogene (*sis*), which encodes an oncoprotein that mimics the 17,000-Da β-subunit of platelet-derived growth factor (PDGF). In fact, the sequence homology of the *sis* oncogene when compared to the PDGF-B subunit is 87%, and the variations that were present could be explained by species variations between humans and monkeys (Doolittle et al., 1983). The PDGF functions to stimulate the proliferation of connective tissue cells (e.g., fibroblasts, smooth muscle cells, and glial cells), and as such the *sis* oncogene is activated in tumor cells arising from connective tissue as opposed to epithelial tissues. Other members of this class of oncogenes include members related to fibroblast growth factors (FGFs), such as *int*-1, *int*-2, *hst*, and *fgf*-5, as well as the related acidic and basic fibroblast growth factors. Relatively little is known of these genes in teleost systems. Both *sis* and *int* were identified in *Xiphophorus* via Southern blots (Anders et al., 1984), and *Wnt*-1 (*int*-1) (Molven et al., 1991) and *fgf*-3 (Kiefer et al., 1996) have been described in the zebrafish (*Brachydanio rerio*).

Class III Oncogenes: Receptors Lacking Protein Kinase Activity

This class includes the *mas* gene, which codes for the angiotensin receptor. To date, neither this nor any other receptor lacking protein kinase activity has been described in any fish models.

Class IV Oncogenes: Membrane-Associated G Proteins

Approximately 20 to 30% of human tumors contain some form of an activated *ras* oncogene, making them the most frequently detected oncogenes in human tumors. Such a high incidence of activated *ras* among tumors of different etiologies points to the critical function of the normal cellular proteins. The *ras* gene superfamily contains over 40 related genes classified into three families: *ras*, *rho*, and *rab* (Downward, 1990). Genes from the *ras* encode small (21-kDa) membrane-bound proteins involved in signal transduction. Activation of c-*ras* is a growth signal for eukaryotic cells, and it is similar to other G proteins in that it binds guanosine triphosphate (GTP). Its GTPase activity catalyzes the conversion of GTP to guanosine diphosphate (GDP). The pathway from GTP to GDP and then to the nucleus is more complicated, involving mitogen-activated protein kinases (MAPKs) and *raf*, passing on the signal to cytoplasmic receptors and an ultimate endpoint in the nucleus. Protein alterations can occur via point mutations, deletions, insertions, or rearrangement through chromosomal translocation, gene amplification, or proviral insertion (Barbacid, 1987); for example, mutations at codons 12, 13, 59, and 61 occur at or near sites of interaction of the *ras* protein with the phosphates of guanine nucleotides.

To date, studies of the *ras* gene involvement during oncogenesis have been reported in at least five species of fish (reviewed in Van Beneden and Ostrander, 1994). Taken in total, it appears that *ras* mutations in fishes are similar to what has been previously reported in mammalian models, including extensive sequence homology and similar mutation spectra; however, only two *ras* genes have been reported in fish as opposed to three in mammals. McMahon and colleagues (1990) examined DNA from liver tumors of winter flounder (*Pseudopleuronectes americanus*) collected from Boston Harbor. Tumor DNA exhibited GC single base changes at codon 12 of flounder c-Ki-*ras*. Likewise, following exposure of rainbow trout (*Oncorhynchus mykiss*) to AFB_1, DNA from 10 of 14 of the induced liver tumors exhibited activating point mutations in the trout c-Ki-*ras* gene (Quintanilla et al., 1991); 7 of the 10 were GGA transversions at codon 12, 2 were GGT transversions at codon 13, and 1 was a codon 12 GGA–AGA transition. In two additional chemical exposure experiments, Lui et al. (2003) reported *ras* gene mutations in medaka (*Oryzias latipes*) following experimental BaP exposure (which included a novel codon 16 mutation and the observation of polymorphic variation within the normal *ras* gene sequence). In a separate investigation, Roy et al. (1999) identified *ras* gene mutations in *Exxon Valdez*

oil-exposed (5.7 g oil/kg gravel) pink salmon (*Oncorhynchus gorbuscha*) embryos. Mutations were seen at codons 12, 13, and 61 of the K-*ras* gene of experimentally oil-exposed embryos. Finally, Wirgin and coworkers (1989) have also reported that an activated K-*ras* exists in tomcod (*Microgadus tomcod*) liver tumors collected from fish residing in areas of the Hudson River that contain high levels of heavy metals, pesticides, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons.

A homolog called N-*ras* has also been reported in zebrafish (Cheng et al., 1997), a model that has primarily found its niche among developmental biologists but also holds potential as a model for carcinogenesis studies. Likewise, Rotchell and colleagues (Nogueira et al., 2006; Rotchell et al., 1995) have reported on the isolation and characterization of the normal *ras* gene from the North Sea flatfish dab (*Limanda limanda*) and European eel (*Anguilla anguilla*) which should enable identification of mutated *ras* alleles in fish collected from waters of high anthropogenic contamination. A *ras*-related homolog called R-*ras* has also been reported in *Rivulus* species (Lee et al., 1998) which suggests that the wider *ras* gene family members are also conserved in fish.

Class V Oncogenes: Cytoplasmic Protein Serine/Threonine Kinases

This family includes the products of the *raf*, *pim*-1, *mos*, and *cot* oncogenes. The best studied is *raf*, which is activated by tyrosine-kinase-associated receptors. Raf protein acts as an intermediary in the signal transduction pathway between *ras* and the cell nucleus by activating the MAPK cascade (Ruddon, 1995). Cytoplasmic protein serine/threonine kinases have not been described to a significant extent in teleost fishes, although, unpublished sequences for a number of these genes in various species appear in GenBank. Detectable expression of *pim*-1 has been reported in both the fish melanoma cell line (PSM) and *Xiphophorus* melanomas (Wellbrock et al., 1998). Likewise, c-*mos* products have been localized via immunocytochemistry, northern blots, and western blots in the testis and epigonal tissue of a dogfish shark (*Scyliorhinus canicula*) (Fasano et al., 1995).

Class VI Oncogenes: Cytoplasmic Regulators

The *crk* oncogene was the first cytoplasmic regulator protein described among oncogenes and acts by stabilizing tyrosine kinases associated with the *src* family of oncoproteins. To date, *crk* has not been reported in any fish.

Class VII Oncogenes: Nuclear Transcription Regulators

Transcription is regulated by the interaction of protein transcription factors with specific regulatory sequences of genes. Transcription factors are comprised of a DNA-binding domain and a *trans*-acting domain which, through protein–protein interaction, enhance binding. A variety of oncoproteins that have been localized to the cell nucleus are known to bind DNA and act as transcriptional activators (Cooper, 1990). Members of this family of oncogenes include, but are not limited to *erb*A, *jun*, *fos*, *myc*, *ets*, *myb*, *ski*, *rel*, *vav*, *maf*, and *pbx*.

A variety of studies have demonstrated that *myc* plays a key role in cell proliferation and differentiation. Activation of cellular *myc* oncogenes (i.e., c-*myc*, L-*myc*, and N-*myc*) has been reported in a number of human cancers (Popescu and Zimonjic, 2002). The rainbow trout (*Oncorhynchus mykiss*) *myc* homolog was one of the first fish oncogenes to be cloned and sequenced (Van Beneden et al., 1986). The investigators subsequently examined liver tumors from medaka (*Oryzias latipes*), lymphomas from northern pike (*Esox lucius*), and liver tumors from white perch (*Morone americana*) via Southern hybridization to trout c-*myc* probes. No rearrangement or amplification of c-*myc* was detected except for a single unique *Eco*Rl restriction pattern in one pike lymphoma sample (Van Beneden et al., 1988). The c-*myc* oncogene has been reported in a number of other fish, including the common carp (Zhang et al., 1995), zebrafish (Schreiber-Agus et al., 1993), and rivulus (*Rivulus ocellatus marmoratus*) (Goodwin and Grizzle, 1994a,b), but little work has been done toward elucidating c-*myc* function during teleost oncogenesis.

The *erb*A oncogene was identified as being an altered version of a thyroid hormone receptor family of DNA-binding proteins and was the first to be identified as a transcriptional regulator (Weinberger et al., 1986). Population- and species-specific restriction fragment length polymorphisms (RFLPs) for

v-*erb*A fragments have been reported in *Xiphophorus* (Zechel et al., 1989). Sequence analysis of two *erb*A homologs revealed two different *Xiphophorus* hormone receptors homologs to the human retinoic acid receptor and the human thyroid hormone receptor.

C-*fos* functions as a transcription factor, and the *fos* oncogene has been reported in osteosarcomas and chondrosarcomas in mice (Finkel et al., 1975). Increased expression of the *fos* oncogene has been reported in DEN-induced hepatic neoplasms from *Rivulus* species (Goodwin and Grizzle, 1994a,b), and c-*fos*-related genes have been induced by neural activation in rainbow trout brain (Matsuoka et al., 1988). A c-*fos*-like protein has also been reported in pufferfish (*Fugu rubripes*) (Trower et al., 1996).

Class VIII Oncogenes: Unclassified

Oncogenes have now been reported among mammalian models that are of unknown function or do not fit a previously define class (e.g., *dbl*, *bcl*-2). To date, although oncogenes of this type undoubtedly exist among fish, they have not been reported; however, methods have been developed to assess the transforming ability of fish tumor DNA via transfection in NIH3T3 cells followed by assessment of tumorigenicity by the nude mouse assay (reviewed in Van Beneden and Ostrander, 1994). This method holds promise for the identification of new oncogenes in fish, as does differential-display polymerase chain reaction (ddPCR) of tumor samples and cDNA arrays.

Tumor Suppressor Genes

Although mutations in oncogenes have been shown to lead to gain of function and activate cell proliferation, tumor suppressor genes lead to a loss of function and act to negatively regulate cell growth and differentiation (Ruddon, 1995). In the simplest form, mutational events (e.g., point mutations and deletions) lead to synthesis of nonfunctioning proteins and as such normal pathways responsible for cell proliferation, growth, and differentiation are inactivated; for example, *p53* acts as a transcription factor, causing cells to be eliminated by apoptosis upon its activation by irreparable DNA damage, overly expressed oncogenes, or many other stresses. Its loss of function may allow a cell to progress inappropriately through cell cycle checkpoints.

The first tumor suppressor gene to be identified was the retinoblastoma tumor suppressor gene and, as discussed below, it was subsequently reported in a number of fish species. Among humans and other mammalian models, significantly fewer tumor suppressor genes have been identified compared to oncogenes (approximately 60), and of these only a few have been reported in fishes. Detailed below are a few of the most frequently studied tumor suppressor genes in teleost models. As was the case for the oncogenes above, this is not intended to be an exhaustive list of those identified in fish, and new ones are being identified on a regular basis. The interested reader is again referred to GenBank. Discussion of a unique oncogene–tumor suppressor gene interaction in *Xiphophorus* is detailed later in this chapter.

Class I Tumor Suppressor Genes: Nuclear Transcription Factors

Retinoblastoma Gene

Retinoblastomas are eye tumors that arise from the retinoblasts along the margins of the developing retina. Among humans, these tumors only occur in the first 1 to 2 years of life before retina development is complete. Mutations in the retinoblastoma tumor suppressor gene (*Rb*) have been found in nearly all retinoblastomas examined and in quite a few other types of cancers, including osteosarcomas, prostate, breast, cervical, lung, and various leukemias (reviewed in Hesketh, 1997). The *Rb* gene product (pl05Rb) is a nuclear phosphoprotein with DNA-binding ability. During the cell cycle, p105Rb remains hypophosphorylated during the early G_1 phase, undergoes sequential phosphorylation as the cell enters S phase, progresses through G_2, and enters mitosis before becoming again dephosphorylated (DeCaprio et al., 1988). Available data suggest that the hypophosphorylated form of the Rb protein may bind with other proteins responsible for DNA synthesis. Thus, alterations in translation or transcription will lead to altered forms of the gene product incapable of binding these proteins and DNA synthesis and ultimately cell proliferation will remain unchecked.

Western blotting has been used to detect the Rb protein in a variety of fishes, including medaka, rainbow trout, English sole (*Parophrys vetulus*), and even the primitive coelacanth (*Latimeria chalumnae*) (Van Beneden and Ostrander, 1994). The complete cloning and sequencing of the *Rb* gene were first published for the rainbow trout (Brunelli and Thorgaard, 1999); however, its potential involvement in chemical carcinogenesis among trout tumor models has not been reported.

The medaka is perhaps the most interesting fish model expressing *Rb*, as retinoblastomas have been induced following exposure of newly hatched fry to methylazoxymethanol acetate (MAM) (Ostrander et al., 1992), making it the only vertebrate model in which retinoblastomas can be induced with regularity. The gene has now been sequenced and mutations have been reported in MAM-induced eye tumors (Rotchell et al., 2001a) and methylene chloride induced liver tumors (Rotchell et al., 2001b). Among feral fishes, the *Rb* gene has also recently been isolated and characterized from the marine flatfish dab (*Limanda limanda*) (du Corbier et al., 2005). Analysis of dab liver adenoma and carcinoma samples revealed *Rb* mutations occurring within the conserved domains of the gene.

p53 Gene

The *p53* gene contains 10 coding exons and is expressed in all types of human cells examined to date, albeit mostly at low levels. Moreover, mutations of *p53* have been reported in more than 50% of all human cancers (Giordano et al., 1998). The human protein is 393 amino acids in length, and a nuclear translocation domain is located near the cyclin-dependent kinase phosphorylation site, which suggests a cell-cycle-dependent signal for *p53* nuclear translocation. Nuclear localization serves an important function for *p53* and is necessary for it to function as a negative regulator of cell proliferation. In some human cancers, it is the inability of *p53* to be transported to the nucleus that is thought to result in excessive cell proliferation. *p53* was the first tumor suppressor gene cloned in fish (Caron de Fromentel et al., 1992), and it was found to exhibit 90% homology to the predicted amino acid sequence of the human gene among five domains. Expression of *p53* has also been reported in catfish (*Ictalurus punctatus*) (Luft et al., 1998), flounder (*Platichthys flesus*) (Cachot et al., 1998), a goldfish epithelioma cell line, and a cell line derived from Chinook salmon embryos via Southern blot analysis (Smith et al., 1988). In an immunohistochemical study, increased expression of *p53* was reported in DEN-induced liver tumors of rivulus (*Rivulus ocellatus marmoratus*) (Goodwin and Grizzle, 1994a,b). Interestingly, studies of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced nonhepatic neoplasms from medaka (Krause et al., 1997) and AFB_1-induced liver tumors from trout (Bailey et al., 1996) have failed to reveal either *p53* mutations or altered gene expression (Krause et al., 1997). Results following an investigation of ultraviolet light inducibility of *Oryzias latipes p53* also suggest that the p53 protein has a different function in lower vertebrates compared with humans (Chen et al., 2001). Recent evidence in support of this comes from the work of Rau et al. (2006), who measured *p53* in a topminnow hepatocellular carcinoma cell line (PLHC-1) and in both immortalized and primary rainbow trout hepatocytes. Their study demonstrated a lack of *p53* induction by a number of classic mammalian inducers (chemotherapeutics), consistent with the idea that piscine *p53* is not regulated in the same manner as human *p53*.

Other Transcription Factors

Other tumor suppressor genes that function as nuclear transcription factors have been reported in mammalian models (e.g., *WT*-1, *E2F*1, *PTC*, *BRCA*-1). To date, there has been a single report of *WT* involvement in a fish model of cancer—evidenced as increased expression in spontaneous nephroblastomas in Japanese eel (*Anguilla japonica*) (Nakatsuru et al., 2000). A *WT*-1 homolog has been found in zebrafish (Kent et al., 1995), pufferfish (Miles et al., 1998), medaka (GenBank Accession No. BAC10628), trout (Brunelli et al., 2001), and carp (*Ctenopharyngodon idellus*) (Wen et al., 2005). A second *WT* gene has recently been identified in zebrafish (Bollig et al., 2006). *E2F* homologs have been identified in zebrafish (Song et al., 2004) and a *PTC* homolog in halibut (*Paralichthys olivaceus*) (GenBank Accession No. BAC57975) and zebrafish (GenBank Accession No. CAB39726). No fish *BRCA*-1 homolog has been reported, although it has been identified in an invertebrate species and is likely conserved.

Class II Tumor Suppressor Genes: Membrane-Bound Signal Transduction Proteins

NF-1 serves as a protypical member of this class. The *NF*-1 gene product is neurofibromin, and it contains a GTPase-activating protein-related domain that is thought to modulate the function of the ras oncoprotein (Xu et al., 1990). Inactivation of *NF*-1 keeps ras in an active ras-GTP state, and the signal for cell proliferation is maintained. Mutations in *NF*-1 have been found in human neurofibromas. Although *NF*-1 has been reported only in the pufferfish (*Fugu rubripes*) (Kehrer-Sawatzki et al., 1998), it may be worthy of further investigation given the number of epizootics of neurofibroma-like etiologies that have been reported in recent years to include neurofibromatosis in the damsel fish (*Pomacentrus paritutus*) (Schmale et al., 1986), chromatophoromas in croaker (*Nibea mitsukurii*) (Kinae et al., 1990), and spindle-cell tumors in the gizzard shad (*Dorosoma cepedianum*) (Ostrander et al., 1995).

Class III Tumor Suppressor Genes: Membrane–Cytoskeleton Interaction Factors

Among mammalian models, a tumor suppressor gene function has been assigned to various proteins involved in membrane cytoskeleton or membrane-extra cellular matrix interactions. *NF*-2, *APC*, *DCC*, and *VHL* are among the best known. For reasons described above, *NF*-2 may be a candidate for further study among specific wild populations exhibiting neurofibroma-like disorders. Recently, a mutation in the *NF*-2 gene was identified as a causal factor in the development of extrahepatic cysts in the bile duct of zebrafish (Sadler et al., 2005), suggesting that the gene is conserved in fish and may be involved in a variety of disease states. Likewise, *DCC*, which has been identified as a heterozygous deletion in about 70% of cases, encodes a protein homologous to neural cell adhesion molecules (NCAMs), and a possible homolog has been described in zebrafish (Holm et al., 1996). It is reasonable to expect that if homologous genes to *DCC* exist in fish they may play a role in carcinogenesis. *APC* homologs have been identified in zebrafish (GenBank Accession No. XP_695239) and carp (*Carassius auratus*) (GenBank Accession No. BAE78584); however, to date no tumor suppressor genes that function as membrane cytoskeleton factors have been fully identified and characterized.

Class IV Tumor Suppressor Genes: DNA Repair Proteins

We are not aware of any DNA repair proteins that function as tumor suppressors that have been identified in fish models, although DNA polymerases, ligases, and other DNA repair enzymes have been described in fishes. The best-studied examples of DNA repair proteins acting as tumor suppressors are found in mammalian systems and include *MSH2*, *MSH3*, and *MSH6*, which encode members of the *MutS* DNA mismatch repair protein superfamily and *MLH1*, *PMS1*, and *PMS2*, which are members of the *MutL*/*HexB* DNA mismatch repair family (Hesketh, 1997). Both the *MutS* and *MutL* superfamilies are seemingly conserved to some degree in zebrafish. (For *MSH2* and *PMS1*, see Yeh et al., 2004, and Lo et al., 2003, and GenBank Accession Nos. NP_998689 and NP_958476, respectively.)

The *Xiphophorus* Melanoma System

The hereditary melanomas of *Xiphophorus* interspecies hybrids, discovered over 70 years ago, represent the best-studied teleost tumor model to date (Schartl and Barnekow, 1982; Zechel et al., 1992). Initially, it was thought that melanoma formation in interspecific F_1 hybrids of the platyfish (*X. maculatus*) and the swordtail (*X. helleri*), which were inherited in a Mendelian fashion, was related to increased expression of the melanin color genes. Subsequent investigations led Anders et al. (1967) to propose that hereditary melanomas in *Xiphophorus* hybrids were the result of the loss of a negative regulator of a specific tumor gene complex (*Tu*). This complex is composed of a pterinophore locus (*Ptr*), which regulates differentiation of this class of pigment cells; compartment-specific loci (R_{co}), which restrict pigment cell differentiation to specific locations; and the melanophore locus (*Mel-Tu*). The latter, if mutated, transforms the melanin-containing pigment cells to neoplastic cells. *Tu* expression in wild-type platyfish was regulated by an unlinked tumor suppressor gene (*Diff*). While swordtails lack both *Tu* and *Diff*, hybrids between these two species are hemizygous for *Tu* and *Diff* and successive backcrosses to the swordtail parent result in progressive elimination of the regulatory genes. This then allows increased

expression of *Tu* and development of malignant melanomas. Although phenotypic expression of *Tu* (as measured by the number and size of spots and the malignancy of the melanomas) highly correlated with elevated expression of the c-*src* gene (Schartl et al., 1985), further studies revealed that *Tu* and *src* were different genes (Maueler et al., 1988).

Restriction fragment length polymorphism (RFLP) analysis of a *Xiphophorus v-erb*B homolog indicated that this oncogene cosegregated with sex chromosome-linked melanoma in *Xiphophorus* hybrids (Adam et al., 1988; Zechel et al., 1988). Partial sequence analysis revealed not one, but two, distinct *Xiphophorus* genes (designated by Anders and colleagues as *x-egfr*A *and x-egfr*B) with homology to the human (*c-erb*B) gene. The most highly conserved region was the tyrosine kinase domain. A nearly full-length cDNA clone was isolated and sequenced from a melanoma-derived cell line and, as with the *x-egfr*B and *x-egfr*A genes above, the clone (designated X*mrk*, for *Xiphophorus* melanoma receptor kinase gene) showed significant homology to the human EGF receptor (Wittbrodt et al., 1989). The Xmrk protein also induces downstream signaling cascades, including *ras*/*raf* and MAP kinase, in a manner similar to the mammalian homolog (Meierjohann et al., 2004). Another downstream interaction identified is with focal adhesion kinase (FAK), which, in turn, triggers pigment cell migration and partly explains the fast metastasis observed in malignant melanoma tumor development (Meierjohann et al., 2006).

The difference in nomenclature adds to the confusion inherent in this system. The *Xiphophorus* melanoma-associated gene related to the human *c-erb*B gene has been designated the *Tu* complex, *x-egfr*B, *x-erb*B*, and X*mrk* (Zechel et al., 1992). Nonetheless, the data are consistent with the interpretation that X*mrk* is the *Xiphophorus* tumor gene *Tu*, a novel receptor tyrosine kinase. Genetic analysis indicated that the oncogenic potential of X*mrk* was regulated by the *Diff* gene, which is postulated to act as a tumor suppressor gene. Transgenic studies involving microinjection of X*mrk* into medaka embryos resulted in embryonic tumors (Dimitrijevic et al., 1988). In addition to overexpression, recent studies have reported that two amino acid changes in the extracellular domain of the protein are also sufficient to convert X*mrk* into an oncogenic version leading to a constitutive, ligand-independent protein (Winnemoeller et al., 2005). *Diff* has been determined to be a member of the CDKN2 family, as revealed by studies of UVB-inducible melanoma in *Xiphophorus* hybrids (Nairn et al., 1996). This is significant in that *CDKN2A* (mammalian homolog *p16*) is a tumor suppressor gene involved in human melanoma.

The *Xiphophorus* model remains perhaps the oldest and best-studied model of oncogene/suppressor gene interaction. Of the model's many unique aspects, the interaction of oncogenes (e.g., x-*erb*B*) and suppressor genes (e.g., *Diff*) is of prime importance. This is currently the only vertebrate system where intimate knowledge of oncogene and suppressor gene interactions and their molecular genetics appears readily accessible. To date, a great many oncogenes and now tumor suppressor genes have been described in *Xiphophorus* compared to other fishes. In addition to those discussed above, reports of *abl*, *src*, *yes*, *fyn*, *erb*A, *fes*, *fgr*, *fms*, *fos*, *int*, *ras*, *myb*, *sis*, *mil*, *myc*, *hck*, *lck*, and *yes* have also appeared in the peer-reviewed literature, and a number of other complete and partial sequences have been deposited with GenBank (Adam et al., 1988; Maueler et al., 1988; Zechel et al., 1988).

Modifying Factors in Chemical Carcinogenesis

Earlier, we described the multiple stages of carcinogenesis, focusing on liver. Factors may modify (enhance or inhibit) carcinogenesis and eventual liver tumor formation by affecting the uptake, transport, and metabolism of the potential carcinogen, leading to changes in DNA adduct formation, or they may affect the promotion or progression of the tumor. When AFB_1, aflatoxicol (AFL), aflatoxin M_1 (AFM_1), and aflatoxicol M_1 ($AFLM_1$) were exposed to trout and the resultant molecular dosimetry (DNA adduction) was established, all aflatoxin adducts except those from $AFLM_1$ were equally tumorigenic (Bailey et al., 1988). In this instance, differences in tumor incidence with the various compounds were largely or entirely accounted for by differences in uptake and metabolism leading to DNA adduction, rather than inherent differences in tumor initiating potency per DNA adduct.

The rainbow trout model has been the principal source of our information regarding modifying factors in fish exposed to chemical carcinogens. Production of hepatic tumors by single chemical agents has been studied in detail, and a great body of evidence has been generated regarding tumor formation and

modulation by factors including steroid hormones, anti- or prooxidants, plant extracts, and environmentally persistent xenobiotics (Bailey et al., 1987b). This is followed, admittedly at some distance, by the medaka. The Oregon State University group (Bailey, Hendricks, and colleagues) has pioneered these efforts, demonstrating a progression of emphasis from *in vivo* bioassays to chemical biodistribution, metabolism, and DNA adduct formation and quantitation to effects on eventual tumor incidence. Their latest work has extended to DNA arrays of resultant tumors and of livers exposed to promotional agents.

Enhancement of Chemical Carcinogenesis: Focus on Trout

The terms *enhancement* and its opposite, *inhibition*, blur the distinction between initiation, promotion, and progression; however, as we shall see it is often difficult to characterize one factor or modulatory compound as having a single effect on the carcinogenetic or tumorigenetic process.

Alteration in Xenobiotic Transport

Pretreatment with selected organochlorines has been found to affect the transport of a carcinogenic PAH *in vivo* (Donohoe et al., 1998). In one investigation, prefeeding with chlordecone or dieldrin was followed by determination of levels of tritiated DMBA (from an aqueous exposure) in stomach and liver. Chlordecone pretreatment did not influence [^{3}H]-DMBA hepatic concentrations, hepatic [^{3}H]-DMBA DNA binding, or hepatic and stomach tumor incidence. It did, however, elevate bile [^{14}C]-CD and [^{3}H]-DMBA concentrations. Dieldrin pretreatment did not influence stomach [^{3}H]-DMBA equivalents or stomach tumor incidence; however it resulted in an elevation in biliary and hepatic concentrations of [^{3}H]-DMBA equivalents. [^{3}H]-DMBA binding to liver DNA was significantly increased and hepatic tumor incidence was elevated by dieldrin pretreatment. This study illustrates enhancement by influence of xenobiotic transport. It is important to note that one organochlorine (dieldrin but not chlordecone) had this effect.

Induced Metabolic Change Resulting in a More Potent Carcinogen

When newly hatched sac-fry were injected with *trans*-7,8-dihydrobenzo(*a*)pyrene-7,8-dioll, hepatic tumors resulted. Marked enhancement was seen with co-injection of BNF or carbon tetrachloride (Kelly et al., 1993a). Perhaps *cyp*-dependent and lipid-peroxidation-dependent pathways could be involved in bioactivation of this compound through epoxidation at the 9,10-position, producing a more potent carcinogen, the (–) enantiomer of BP-7,8-DHD (Kelly et al., 1993a). Prior treatment of trout with BNF increased O^6-ethylguanine formation by subsequent DEN exposure, enhancing the initiation phase of carcinogenesis (Fong et al., 1988). Another example of increased DNA adduct formation and enhanced tumorigenesis was provided in MNNG studies (Kelly et al., 1993b). Dietary hydrogen peroxide enhanced levels of the mutagenic DNA adduct 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) and tumor formation.

Synergistic or Cocarcinogenic Enhancement

Both cyclopropenoid fatty acids (CPFAs) and aflatoxin are complete carcinogens in the trout model. Simultaneous exposure to cyclopropenoid fatty acids and to aflatoxin (Lee et al., 1968) revealed a synergistic effect (cocarcinogenesis) on liver tumor formation in rainbow trout; however, subsequent work by Bailey and colleagues (1982) suggested that dietary CPFAs repress *cyp* activities and depress DNA damage by AFB_1 *in vitro*. Thus, two factors may be at work in chronic exposures: depression of initial AFB_1-induced DNA damage but highly efficient promotion of transformation from the remaining lesions, resulting in a synergistic effect. Bailey's group (1987b) also provided another example of cocarcinogenesis by simultaneous treatment of trout with PCB (Arochlor® 1254) and DEN. Co-administration led to synergism of the tumor response.

Promoters of Tumorigenesis

When trout are exposed to BNF or indole-3-carbinol (I3C) from cruciferous vegetables *after* AFB_1 initiation, a significant enhancement of tumor response occurs (Bailey et al., 1982). These compounds do not induce tumor formation when given alone; therefore, they are promoters of tumorigenesis. As

we shall see later, however, the same compounds have inhibitory effects on AFB_1, DEN, and PAH liver carcinogenesis when administered *prior* to or concurrent with initiation (Bailey et al., 1982, 1987b, 1991; Dashwood et al., 1991). In an effort to gain a more firm understanding of the promotional property of I3C, 9000 trout embryos were initiated with AFB_1 (concentration ranges 0 to 250 ppb) by 30 minutes of aqueous immersion (Oganesian et al., 1999). Subsequently, these groups were fed experimental diets with I3C (range of 0 to 1250 ppm). The findings supported multiple mechanisms for I3C in promotion. I3C proved estrogen-like inducing vitellogenin at the lowest dietary level. This is not surprising, as estrogens are known to promote hepatocarcinogenesis in trout (Nunez et al., 1989) and medaka (Cooke and Hinton, 1999). CYP1A was induced through the aryl hydrocarbon receptor (AhR) pathway at a dietary I3C level of 1000 ppm and above.

When extracts prepared from oil refinery effluents were tested for carcinogenic potential, with and without exogenous rat S-9 activation, no neoplasms were detected; however, extracts co-injected with AFB_1 induced elevated frequencies of hepatic neoplasms (Metcalfe and Sonstegard, 1985). This reveals a synergistic effect of the extracts on AFB_1-induced liver carcinogenesis in the trout model.

Carcinogen bioassays may indicate enhancement by unknown agents as in undefined mixtures (Hendricks et al., 1980). An example of this was provided in studies with glandless cottonseed kernels that are free of one cyclopropenoid fatty acid, gossypol, but still contain naturally occurring cyclopropenoid fatty acids. The latter are active as synergists with aflatoxins and are primary liver carcinogens. Diets containing glandless cottonseed kernels or a lightly processed cottonseed oil produced significant numbers of hepatocellular carcinomas in trout after 1 year. The much greater incidence of cancer induced by the kernel than by the oil indicates that synergists or other carcinogens may be present in the kernel in addition to the cyclopropenoid fatty acids (Hendricks et al., 1980).

Fumonisin B_1 (FB_1), a mycotoxin, is a liver carcinogen in rodents. FB_1 was not a complete carcinogen in trout (Carlson et al., 2001). Instead, FB_1 promoted AFB_1-initiated liver tumorigenesis. In MNNG-initiated fish, only liver tumors were promoted; in fact, tumor incidence decreased in kidney and stomach. This illustrates the inherent difficulty in characterizing a single agent for its overall enhancing or protective effect in carcinogenesis. Moreover, this study further demonstrated that differences may be seen with different initiators and at different tumor sites, depending on the time of administration. In addition, species-specific differences will likely make generic classification difficult if not impossible.

Dehydroepiandrosterone (DHEA), the most abundant steroid secreted by the adrenal glands, can also be converted into other steroid hormones, including testosterone and estrogen. DHEA is a carcinogen in rodent models and, when studied in the trout, proved to be a complete liver carcinogen and potent tumor promoter of AFB_1 carcinogenesis (Orner et al., 1995). Interestingly, the tumorigenicity of DHEA has been thought to follow its peroxisomal proliferative activity; however, the trout study showed that the tumor end stage was reached without evidence for peroxisome proliferation and provided the initial evidence (mutation of Ki-*ras*) that DHEA (Orner et al., 1995) could be a genotoxic carcinogen. Subsequent studies with the direct-acting carcinogen MNNG examined the effects of post-initiation exposure to DHEA (Orner et al., 1996). Using trout fry, MNNG was administered as a 30-minute bath exposure, and later these same trout were fed diets containing various concentrations of DHEA. This protocol led to a dose-dependent increase in liver tumors (multiplicity of neoplasms and their size). Kidney tumors were also enhanced; however, the total number of stomach and swim bladder tumors was reduced by DHEA treatment. This study demonstrated the differential effects of DHEA on MNNG-initiated carcinogenesis and, with the power of this multi-organ model, indicated that effects on all resultant neoplasms must be considered. Because DHEA was proven to be a complete hepatocarcinogen in the trout, its enhancing effects on AFB_1 and MNNG must be considered cocarcinogenic or synergistic; that is, the first carcinogen may have caused initiation or some foci of cells that was resistant to the toxic effect of the second carcinogen and thereby allowed for the preferential expansion of the foci to large growth with a decreased time to tumor formation. Orner et al. (1998) demonstrated that the latency period for aflatoxin-induced hepatocarcinogenesis was reduced by administration of DHEA after AFB_1. These investigations also revealed that DHEA may act through alterations in cell-cycle control and that serial bioassay is useful in determining the mechanistic factors through which the cancer phenotype may be achieved.

Shelton and associates (1984) fed trout diets containing the complete carcinogen DEN, with and without Arochlors 1242 or 1254. Both Arochlors enhanced DEN liver tumor incidence. As will be shown below, this is in contrast to earlier studies with AFB_1 that showed an appreciable inhibition of liver tumor incidence. The direction of the modulation of chemical carcinogenesis in trout by PCB depends on the carcinogen involved.

Partial hepatectomy is a proven way to induce liver regeneration and has been used to enhance liver tumorigenesis in rodents (Michalopoulos, 1995) and in medaka (Kyono-Hamaguchi, 1984). The liver rapidly regenerates following this procedure, and, if the carcinogen is administered prior to partial hepatectomy, the initiated cells preferentially respond to the growth stimulus, thereby reducing the latency period for tumor formation (promotional effect). The cytotoxicity phase following diethylnitrosamine exposure was studied in medaka using light microscopy, electron microscopy, and biochemical indices (Lauren et al., 1990). Perhaps the toxicity serves as a sort of chemical hepatectomy and plays an enhancing role in the eventual DEN-induced liver carcinogenesis in this model. Although the technique has yet to be utilized in chemical carcinogenesis studies, Ostrander and coworkers (1993) developed a partial hepatectomy procedure for both large (2 kg) and small (<5 gm) trout.

In recent bioassays using large numbers of larvae initiated by a brief, aqueous DEN exposure, tumor frequency was consistently higher in females than in male medaka (Teh and Hinton, 1988). This interesting finding was the impetus for several studies examining features of tumor growth in female and male medaka using a two-step, initiation-promotion assay in which a subcarcinogenic dose of initiating carcinogen was followed by continuous administration of a tumor promoter. The hypothesis that estrogen is a promoter of hepatic neoplasia in medaka was tested, and results demonstrated that E_2 increased the prevalence of hepatocellular adenoma and carcinoma, as well as basophilic hepatic foci of cellular alteration (FCA) (Cooke and Hinton, 1999). The effects of steroid hormones on normal liver physiology suggest that endogenous estrogens may contribute to the increased susceptibility of female fish to hepatic neoplasia. Interestingly a similar response was observed with a nonsteroidal estrogenic compound, β-hexachlorocyclohexane (βHCH), which is not a strong ER agonist, suggesting that multiple mechanisms may exist for tumor promotion putatively involving membrane initiated signaling (Cooke and Hinton, 1999).

Enhancement of Chemical Carcinogenesis: A Focus on Medaka

One feature common to laboratory and field studies with various fish species is a higher prevalence of hepatocellular neoplasia in females than in males. The medaka (*Oryzias latipes*), a small aquarium teleost fish, is a highly valuable vertebrate model for hepatocarcinogenesis because of its small size, ease of maintenance, rapid tumor onset, and immense potential as a bioassay subject (Hawkins et al., 1995). Factors that appear to positively modulate tumorigenesis in medaka are sex, estradiol, and temperature.

Sex

Females have briefer latency periods and greater tumor volume. Control medaka liver morphology proved highly correlated with sex and this in turn was correlated with ovarian maturation. Sex-specific aspects of individual medaka and liver growth were serially investigated following repeated observations that females were more susceptible to hepatocarcinogenesis when initiation was performed within the first 3 weeks after hatching (Teh and Hinton, 1998). Aspects of sex-specific growth in hatchling, immature, and sexually mature control medaka were determined. For each fish, body and liver weights were recorded, and the ratios of liver weight to body weight (hepatosomatic index [HSI]) were determined. Body weights of control females were significantly greater than those of males at weeks 8, 20, 32, and 44. Liver weights and HSIs were significantly greater in females vs. males at all ages. Similar recordings were made for medaka initiated with a 48-hour aqueous exposure to 250 ppm DEN. Among the DEN-treated medaka, female body weights were significantly greater than their male counterparts at weeks 8, 16, 20, and 32. Female liver weights were greater than male values at all weeks except for 4 and 6, and female HSIs were significantly greater than male values at all times. A higher incidence of FCA (40%) distinguished females from males (10%) at 4 weeks, and these values reached 100% incidence (females) and 90% (males) at week 12. Tumor latency periods for adenomas and carcinomas were

significantly shorter in females than males. At week 20, the incidence of tumors was significantly higher in females than in males. Results indicate that sex-specific differences appear in body weight, but especially liver weights and HSIs as a function of larval development, ovarian maturation, and age, in control and DEN-treated medaka. Tumor incidence and time to endpoint (latency period) demonstrated female growth to be a promotional stimulus, positively modulating DEN hepatocarcinogenesis.

Estradiol

Feeding of estradiol (E_2) after initiation proved to be a promotional stimulus as seen in preferential growth of basophilic foci of altered hepatocytes, diminished latency period, and increased incidence of hepatocarcinogenesis in males vs. controls receiving carcinogen alone (Teh and Hinton, 1998).

Temperature

The temperature of the surrounding water was elevated during promotion and progression phases of hepatocarcinogenesis, which led to a marked increase in gonadal and hepatic weights in females with resultant positive modulation of hepatocarcinogenesis. Hepatocytes of mature females (≥3 yolked oocytes) were deeply basophilic, with a distinct purple coloration consistent with vitellogenin (yolk precursor) synthesis which is known to follow estradiol stimulation (Teh and Hinton, 1998). Cooke and Hinton (1998) also tested the hypothesis that estrogens, either natural or xenobiotic, promote growth of hepatic preneoplastic lesions and tumors. Medaka were exposed to a low dose of the carcinogen DEN at 3 weeks of age, then fed a purified casein-based diet or the same diet with E_2 or the xenoestrogen β-hexachlorocyclohexane (β-HCH) daily from 4 weeks to 28 weeks of age. Histopathologic analysis revealed that E_2 increased the incidences of hepatocellular adenoma or carcinoma. With increasing dietary concentration of E_2, average numbers of basophilic foci rose and numbers of eosinophilic foci decreased. Treatment with β-HCH raised the incidences of tumors and basophilic foci. E_2 particularly promoted tumor development in male medaka, indicating that xenobiotics with mechanism of action like that of E_2 may escalate growth in feral fish of previously initiated cells into tumors.

Inhibition of Chemical Carcinogenesis

When fed simultaneously with AFB_1, PCB (Arochlor® 1254) produced an inhibitory effect on liver tumor incidence (Hendricks et al., 1977; Shelton et al., 1984). In the former study, liver tumor incidence was inhibited and the degree correlated with the level of PCB in the diet. The inhibition in tumor response was apparently not due to growth retardation by the modulator as AFB_1, but not Arochlor® 1254, diminished growth. It is possible that some of the effect may have been related to activation of AFB_1 as liver cytosolic fraction from these fish proved less capable of converting the compound to a mutagen in the Salmonella mutagenesis assay (Shelton et al., 1984).

Modulators (PCB, BNF, and I3C) of cancer in conventional experimental animals were tested in the trout model for their ability to inhibit or reduce tumorigenesis (Bailey et al., 1982; Nixon et al., 1984), and their mechanisms of inhibition of AFB_1 carcinogenesis were investigated. PCB and BNF, but not I3C, are known as strong inducers of trout cytochrome CYP1A and associated activities (Bailey et al., 1982). Whereas BNF induced *cyp* in a dose-dependent manner, I3C had no effect *in vitro*. Dietary induction by BNF or PCB was shown to be accompanied in isolated hepatocytes by considerably altered AFB_1 metabolism and by significantly reduced rates of DNA adduct formation for all three agents. All three agents differentially altered *in vivo* AFB_1 pharmacokinetics, enhanced bile elimination of AFB_1 as the aflatoxicol-M_1 glucuronide, and significantly reduced peak levels of liver DNA adduct formation. When the compounds were incorporated in diet and fed before or during AFB_1 exposure, BNF and I3C proved most responsive in inhibition of liver tumor incidence. Whereas BNF induced *cyp* in a dose-dependent manner, I3C had no effect *in vitro*. Both BNF and I3C reduced AFB_1–DNA binding *in vivo* and provided marked protection against AFB_1-induced hepatocarcinogenesis (Bailey et al., 1982). These data demonstrated that induction of *cyp* was not necessarily required for alterations in DNA adduct formation *in vivo* or protection against AFB_1 carcinogenesis. These findings suggest also that enhancement and inhibition may occur due to actions at one or more of various sites along the tumor-forming

process; for example, BNF may alter *cyp*, increasing detoxification and reducing initial DNA damage by AFB_1. Finally, I3C may alter AFB_1 pharmacokinetics.

As has been presented above, DEN is a complete carcinogen in the trout and medaka models. Inhibition of the DEN-induced hepatocarcinogenesis was demonstrated using I3C (Fong et al., 1988). To test for possible inhibitory effects on DEN-induced hepatic tumors, trout were prefed a diet containing BNF, I3C, or Arochlor® 1254 and, following a recovery period, were exposed to a tumorigenic concentration of DEN in water for 24 hours. Fish were allowed to grow for 42 weeks and then assessed for liver tumor formation. Liver DNA ethylguanine levels were reduced in I3C-pretreated fish, were increased in BNF-pretreated fish, and showed no significant effect with Arochlor® 1254. The authors suggested that I3C inhibition was mediated by effects on O^6-ethylguanine formation and, therefore, on the initiation phase of carcinogenesis.

Chlorophyllin (CHL), a food-grade derivative of the green plant pigment chlorophyll, proved to be a potent, dose-responsive inhibitor of AFB_1–DNA adduction and hepatocarcinogenesis in the trout model (Breinholt et al., 1995a). These effects occurred when CHL was fed in a diet containing AFB_1. When CHL was fed after exposure to AFB_1, it neither enhanced nor suppressed AFB_1 liver tumorigenesis. These findings suggest that CHL interferes with early events in AFB_1 carcinogenesis. A subsequent *in vitro* study (Breinholt et al., 1995b) revealed that CHL formed a strong noncovalent complex with AFB_1 and inhibited phase I metabolism. A third study by Breinholt et al. (1999) focused on the complex formation and *in situ* protective mechanisms within the target organ, liver. Bioavailability differences rather than *in situ* organ inhibitory mechanisms are likely the cause of the protection afforded by CHL. Finally, the cancer prevention properties shown by CHL against AFB_1 were tested using another class of carcinogen, the PAHs (Harttig et al., 1996). Dibenzo(*a*,*l*)pyrene (DB(*a*,*l*)P) is the most carcinogenic of the PAHs tested in the trout model to date. Features include the occurrence of tumors of liver, stomach, and swim bladder. Use of this compound provided the opportunity to determine whether CHL would be effective in inhibition of multi-organ carcinogenesis. CHL reduced DB(*a*,*l*)P toxicity, inhibited DB(*a*,*l*)P–DNA adducts, and strongly inhibited DB(*a*,*l*)P mutagenesis in the *Salmonella* assay.

As we have seen, inhibition of chemical carcinogenesis, or cancer chemoprevention, has been studied only in the trout model. Each of the examples presented herein suggests that the protection occurs by inhibition of early events, including xenobiotic uptake or distribution and alteration in phase I metabolism favoring detoxification. The final result of these changes is a net reduction in carcinogen–DNA adduction. This does not mean that other mechanisms are not operative in later stages, as between initiation and promotion or progression; they simply have not been addressed. Work to date has not considered proliferation, differentiation, or cell death. As presented above, only one paper has included serial analysis with emphasis on factors governing the cell cycle (Orner et al., 1998). The general approach has been to alter early events reducing DNA adduction and then to determine the effect of such alteration on eventual tumor incidence. What is now increasingly possible and is a critical challenge is a careful analysis during pathogenesis of fish neoplasia incorporating self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Immunologic Factors

The immune system and its role in the carcinogenesis have been studied in mammalian models, although parallel studies in fish models have yet to be conducted. Regulatory T (T-reg) cells are an important component of the immune system that were implicated in cancer progression as early as 1974 (Umiel and Globerson, 1974). Subsequent work during the 1980s focused on the disruption of T-reg cells and attempted to induce tumor immunity (Orentas et al., 2006). It is the functional inhibition of such cells that is currently the goal of effective anticancer immune therapies in humans. To date, only a limited number of CD4 T-cell gene homologs have been isolated from fish, including pufferfish (*Fugu rubripes*) (Suetaka et al., 2006), trout (*Oncorhynchus mykiss*) (Dijkstra et al., 2005), sea lamprey (*Petromyzon marinus*) (Pancer et al., 2004), and channel catfish (*Ictalurus punctatus*) (GenBank Accession Nos. ABD93355 and ABD93354). It is noteworthy that the CD4 homologs characterized thus far display many of the structural motifs found in mammalian counterparts. An exception is a particular motif

present in humans but not the primitive fish sea lamprey (Pancer et al., 2004). The overall homology is sufficient, however, for the authors to suggest that it is an ancestral CD4-like gene. Future work might potentially involve an investigation into the expression of CD4-like genes in fish displaying normal, adenoma, and carcinoma phenotypes to determine if it is indeed involved in modulating the progression stage of the carcinogenesis process. It is only a matter of time before recent advances in -omics technologies highlight the involvement of CD4 and other immune response elements in modulating cancer in fish.

Environmental Factors

All fishes live in water, which, as a universal solvent, brings them into direct contact with a variety of waterborne substances, many of which are anthropogenic contaminants. In addition, fishes are exposed through diet to substances that are not readily soluble in water but have been accumulated within the tissues of the organisms on which fish feed. Fishes have also developed a variety of physiological adaptations to poikilothermy, where body temperature is dependent on ambient temperature, and unique morphologic adaptations to aquatic life, largely in response to the high density and low compressibility of water. Some fishes produce large eggs that can be easily examined and manipulated outside the body, and some demonstrate what researchers using mammalian models might call "unusual" reproductive strategies, such as the physical transition of some female wrasses (family Labridae) to the role of dominant male when the need arises. Other fishes undergo radical physiological adaptations associated with life-stage transitions, exemplified by the metamorphosis of the lamprey (*Petromyzon marinus*) and the process of smoltification, an array of morphologic, physiologic, and behavior adaptations for ocean life that occurs among most salmonids. In searching for an ideal mammalian experimental model, researchers often try to identify animals that express disease processes homologous to those seen in humans. The same strategy has been applied to fish models to variable extent; however, fish may prove to be better models in many circumstances, not due to their similarity with humans or other mammals but precisely due to their differences. This can be further amplified when one considers the ability of environmental factors to modulate carcinogenesis.

Complex Mixtures

Complex mixtures are an important factor, especially when we deal with field studies and when extracts of environmental media (sediment and industrial mixtures) are used (Fabacher et al., 1991; Metcalfe and Sonstegard, 1985). An excellent example of complex mixtures was provided by Gardner et al. (1998). A chronic 6-month carcinogenicity bioassay was conducted on-site in a mobile biomonitoring facility using medaka in an initiation–promotion protocol, with DEN as the initiator and trichloroethylene (TCE)-contaminated groundwater as the promoter. TCE (reagent-grade) was added to carbon-filtered groundwater to simulate concentrations found in contaminated groundwater. Although the filtered water with TCE showed no promotional effects, a tumor-promoting effect was seen in the unfiltered groundwater after initiation with DEN. This implies that substances in the groundwater and not detected by chemical analysis were working to promote DEN carcinogenesis.

Complicated Exposure Regimes

Field investigations are difficult to interpret in part due to complicated exposure regimes, the environmentally realistic scenario. Variations in metal content in surface waters, with higher levels being present during rainy seasons, represent a portion of the complexities with fieldwork. This could lead to differences in tumor incidence due to promotional effects related to formation of active oxygen intermediates. Intermittent exposures may lead to variations in the promotion or inhibition of earlier initiated carcinogenic processes and thereby lead to errors in the potential for fish living at a particular site to develop tumors. With methylazoxymethanol acetate (MAM) exposure, a similar incidence of liver tumors (i.e., nearly 100%) was found after a long-time exposure to a low level (0.1 ppm for 120 days) and after a short exposure to a high level (10 ppm for 1 hour) of the initiating carcinogen (Aoki and Matsudaira, 1984). Dose-dependent enhancement and inhibition of the tumorigenic process were reviewed earlier.

Importantly, the responses of fish to their environment integrate the processes of uptake, distribution, bioavailability, metabolism, degradation, and the combinatorial effects of complex mixtures (Long and Buchman, 1990).

Temperature

Fishes are poikilothermic, with body temperatures that fluctuate with ambient temperatures. Because they are poikilotherms, it has been possible to analyze the effect that changes in temperature exert on carcinogenesis in medaka (Kyono, 1978; Kyono and Egami, 1977; Kyono et al., 1979; Kyono-Hamaguchi, 1984). Following exposure to DEN for 6 to 8 weeks at 25°C, liver tumors appeared after 11 to 13 weeks. The greatest effect on tumorigenesis was seen when fish were initiated and allowed to grow out at high temperature (25°C). When the initiation temperature was reduced to 5 to 8°C, this resulted in no tumors; however, for grow out after initiation at a low temperature (25°C), a few tumors resulted. Labeling and mitotic indices were performed on the above groups, and the results showed the highest rate of DNA synthesis and cell divisions at high initiation and high grow-out temperatures.

Similar results were seen in trout, and additional mechanisms were investigated (Curtis et al., 1995). First, trout were acclimated at one of three temperatures—cool (11.0°C), warm (18.0°C), or intermediate (14.5°C)—for 1 month. Next, they were exposed to AFB_1 for 30 minutes and then reared for 9 months. Tumor incidence and multiplicity tracked increasing temperature. In addition, downward temperature shifts reduced AFB_1–DNA adduction. In a follow-up study (Zhang et al., 1992), trout were acclimated at one of three temperatures and then immersed in [^{3}H]-AFB_1 at their respective acclimation temperatures or at higher temperatures. The total radioactivity was used to establish uptake. DNA adducts were less persistent in fish maintained at 21 days at 18°C than at 10°C. These findings suggest that temperature modulation of AFB_1 genotoxicity occurs by three mechanisms: uptake by liver and the formation and persistence of resultant DNA adducts (Zhang et al., 1992). In the most recent trout temperature studies (El-Zahr et al., 2002), the PAH DMBA was administered in a single exposure (20-hour duration) to trout previously acclimated at 10, 14, or 18°C. Various temperature shifts were used to assess the effects on hepatic uptake, DNA adducts, and eventual tumor incidence. When fish were shifted to higher temperature after a 4-hour DMBA exposure, DMBA in liver increased with temperature. In fish exposed to 14°C and then shifted to 10°C or to 18°C for 3 days, adduct levels were higher in the former. After 21 days, adduct persistence was less in fish at 18°C than those at 10°C. After 9 months' rearing, the incidence of stomach, liver, and swim bladder tumors showed a dramatic increase with increased rearing temperature. The investigators considered the loss of persistence of DNA adducts with higher temperatures to be evidence for increased error-prone DNA repair at warmer temperature (El-Zahr et al., 2002).

Crowding/Stocking Density

One of the most important advantages of aquarium fishes such as medaka is that large numbers of animals can be reared in small indoor aquaria; however, no standardized protocol exists that provides stocking density guidelines for carcinogenesis studies, and, indeed, stocking rates are frequently not stated in published reports. Stocking density, which has been shown to have significant effects on growth in other fish species (Canario et al., 1998; Chua and Teng, 1979), was reported infrequently in carcinogenesis studies published between 1982 and 1999 utilizing medaka as test animals (Davis et al., 2002). Stocking densities commonly used in carcinogenesis assays can significantly affect growth rate and fecundity in medaka (Davis et al., 2002). In light of the interrelationships of food restriction (Avula et al., 1999; Zhu et al., 1999) and steroid hormones (Degen and Metzler 1987; Virna et al., 1996) with cancer, stocking density, with its effect on growth and reproduction, can potentially impact carcinogenesis study outcomes. As an added factor, stocking density can impact water quality, depending on the type of aquarium system used. Significant reductions in dissolved oxygen and increases in total ammonia nitrogen have been reported with increasing the stocking density of palmetto bass kept in aerated outdoor ponds (Liu et al., 1999). Also, body weight and daily egg production were found to be further reduced when, with stocking density held constant, the feeding rate was reduced from excessive feeding (20% of body weight daily up to 8 weeks of age, then 15% of body weight daily thereafter) to mildly restrictive (10 to 12% of body weight daily up to 6 weeks of age, then 5% of body weight daily thereafter) (Davis

et al., 2002). This study showed that the basic husbandry practices of stocking density and feeding rate significantly impact growth and egg production in female medaka.

Host Factors in Chemical Carcinogenesis

We have seen that environmental abiotic factors such as the temperature, type of carcinogen, duration of exposure, time of exposure onset, etc., can drastically impact the expression of tumors in fishes. Conversely, biotic factors of the fish also appear capable of playing a significant role in carcinogenesis in fishes.

Sex

As discussed earlier, significant body and liver weight differences between male and female medaka have been observed (Teh and Hinton, 1998), with females growing at a faster rate than males and achieving a greater liver weight than their male counterparts. In addition, differences were found in tumor incidence (females higher) following carcinogen (DEN) exposure (Cooke and Hinton, 1999; Teh and Hinton, 1998). Similar sex-specific results were seen when Reddy et al. (1999a) exposed medaka to dibenzo(*a*,*l*)pyrene. Life-span studies were conducted on spontaneous tumor development in medaka in outdoor culture ponds (Masahito et al., 1989). The data indicated a particular susceptibility of older female medaka to liver tumor, but not to any other type of tumor development. Incidence was higher in females than in males from 3 to 5 years of age, reaching 7.1% in 5-year-old female stock (Masahito et al., 1989). The fact that these studies showed clear sex differences (females were more responsive) when others using the same carcinogen (DEN) have not (Brown-Peterson et al., 1999) may be due to differences in husbandry practices, the genetic background of medaka, and the compound to which they were exposed. Toussaint et al. (1999), for example, using a chronic bioassay, exposed medaka to a complex environmental mixture (contaminated groundwater) after initiating the fish with DEN. Treatment resulted in greater tumor incidence in male than female fish (Kissling et al., 2006). Propanediol, when fed, proved to be a multisite carcinogen in both sexes of rats and mice, but it caused increased liver tumors only in male guppies and male medaka (Gunnels et al., 2005).

The same husbandry factors that affect growth may also affect the onset of sexual maturation and reproductive performance. Results from the stocking density study reviewed above demonstrated significantly lower body weights and daily egg production in female medaka reared at high vs. low stocking densities (Davis et al., 2002). Also, body weight and daily egg production were further reduced when, with stocking density held constant, the feeding rate was reduced from excessive feeding (20% of body weight daily up to 8 weeks of age, then 15% of body weight daily thereafter) to mildly restrictive (10 to12% of body weight daily up to 6 weeks of age, then 5% of body weight daily thereafter). The Davis et al. study (2002) demonstrated that the basic husbandry practices of stocking density and feeding rate significantly impact growth and egg production in female medaka. Interestingly, stocking density or feeding rate did not significantly affect male body weights; thus, husbandry practices may influence males and females differently, and, to the extent that sex factors may influence study outcome, husbandry factors are important sources of variability. It is possible that sex-associated differences may be masked because of the common practices of heavy stocking in combination with low feeding rates. Under such conditions, females may not have adequate space and food for maximal growth and reproductive development (Davis et al., 2002).

Species Differences

Species-specific differences are frequently observed between fishes, rodents, and higher mammals, including humans. As our review of the trout model has shown, these vertebrates are particularly sensitive to AFB_1; however, in monkey and human liver preparations, the major microsomal biotransformation product of AFB_1 is aflatoxin Q_1, and this product proved 100 times less carcinogenic than AFB_1 in the trout model. These studies demonstrated species differences in the metabolism of the procarcinogen (Hendricks et al., 1980). Trout exhibit only limited capacity for DNA repair, especially for removal of bulky DNA adducts (Bailey et al., 1988).

Inhalation exposure of nitromethane proved to be a strong carcinogen in conventional models, as it caused mammary gland tumors in female rats, Harderian gland and lung tumors in male and female mice, and liver tumors in female mice. After a different route of exposure, however, it failed to increase tumors in either guppies or medaka (Kissling et al., 2006). Another strong carcinogen in conventional models, 1,2,3-trichloropropane, did not result in a strong response with two fish species. When administered by oral gavage, 1,2,3-trichloropropane was a multisite carcinogen in both sexes of rats and mice and caused an increased incidence of tumors in the liver of both male and female guppies and medaka and in the gallbladder of male and female medaka. Compared to trout, zebrafish showed a fourfold less adduction of the carcinogen AFB_1 to liver DNA (Troxel et al., 1997b), and they proved relatively resistant to dietary AFB_1 hepatocarcinogenicity.

Fishes adapted for rearing in a laboratory situation have been proposed as surrogate species to study the impact of a variety of environmental changes on indigenous, possibly threatened or endangered, fishes. A fish model is really the only appropriate model for such surrogate studies; however, the selection of an appropriate model must be done with an eye toward individual species environmental requirements and physiological profiles. Due to the great diversity of this vertebrate group, different fishes may vary by several orders of magnitude with regard to acute sensitivity to environmental contaminants (Elonen et al., 1998; Geyer et al., 1993). Rainbow trout (*Oncorhynchus mykiss*) and Coho salmon (*Oncorhynchus kisutch*) were exposed to AFB_1 either by passive embryo uptake or by dietary treatment after hatching and onset of feeding. Trout exposed as embryos to an aqueous solution of 0.5 ppm AFB_1 for 15 minutes showed a 62% tumor incidence 12 months later that proved to be nearly 7-fold greater than the tumor incidence of the Coho salmon despite the fact that they were exposed for twice as long (Bailey et al., 1988).

Finally, organ inhomologies exist between various fish and mammals and among fishes. As Bailey and coworkers (1996) have pointed out, fish lack mammary glands, cervical epithelium, salivary glands, and a definitive colon and by no means is the gill equivalent to the lung with its tracheobronchial epithelium—all of which are major targets for cancer development. As such they would not serve to model these processes. However, there are also basic anatomical considerations among fishes and these should not be overlooked. Trout, but not medaka and zebrafish, are gastric teleosts. As we have seen earlier in the chemoprevention section, it is the I3C acid condensation products and not the parent compound (Dashwood et al., 1994) that affords protection against AFB_1-induced tumorigenesis in the trout. Whether protection would be afforded by 13C in agastric teleosts is an interesting question.

Diet

Trout were fed a range of dietary components that altered their carcinogenic response to AFB_1 (Bailey et al., 1987a). Dietary protein at levels substantially exceeding nutritional requirements was synergistic with AFB_1. Cyclopropene fatty acids (CPFAs) were carcinogenic when fed alone at 20 or 55 ppm and synergistic when fed with AFB_1. High dietary protein is a synergist for AFB_1 carcinogenesis (Bailey et al., 1982), apparently enhancing the transformation of the procarcinogen to the form initiating DNA adduction and damage. Fish treated with AFB_1 as embryos and then reared on high-protein diets had substantially higher incidences of hepatocellular carcinoma than similarly treated trout fed normal protein diet or high protein controls without AFB. Of interest would be determining whether the growth of trout differs with alterations in dietary composition, as this may be a factor in modulation of liver tumor production due to the fact that a liver-to-body weight ratio is maintained. If the body weight increases so would the liver weight, and increased hepatic growth would likely promote tumor development. Earlier studies in trout (Lee et al., 1978) addressed this question. Diets containing either 49.5% or 32% casein or fish protein concentrate (FPC) and containing a range of 5 levels of AFB_1 were fed to young rainbow trout for 12 months. Fish consuming diets with the FPC as protein source had greater growth and a higher tumor incidence compared to those consuming the casein-based diets. AFB_1 exposure reduced growth, and the fish consuming the diet with only 32% casein showed less growth. Because procarcinogens are also acute liver toxicants, the added growth afforded by FPC as a dietary source likely served as a promotional stimulus that affected initiated cells preferentially.

Life-Stage Effects

The earliest attempts to create tumors in fish were by exposing adult individuals to suspect or reference carcinogens (Sinnhuber et al., 1968; Stanton, 1965). Because tumorigenesis is a chronic form of toxicity, most investigators have since moved to early-life-stage exposures. With few exceptions, exposure as embryos or larvae (newly hatched sac-fry trout) works well, and apparently initiation is followed by periods of rapid growth, further promoting the tumor-forming process. In one study (Kelly et al., 1993a), when trout embryos were injected with pure enantiomers of *trans*-7,8-dihydrobenzo(*a*)pyrene-7,8-diol, high mortality resulted. Subsequent efforts waited until microinjection of newly hatched sac-fry could be performed. Using this protocol, mortalities were reduced and tumors resulted. This suggests that, under these conditions, embryos were more sensitive to the acute toxic effects of the compound.

The Oregon State University group first used bath exposures of embryos, and under these conditions, usually involving exposure duration of 30 minutes, older embryos (closer to hatching) proved more sensitive to AFB_1. Advantages included use of a small amount of compound for testing and dose–response relationships were obtained (Hendricks et al., 1980a). In a 1984 review (Hendricks et al., 1994), more results and details of the embryo exposure were provided. The embryos used were 21 days old (normally hatch at 24 to 25 days) and when exposed to AFB_1 were sensitive to both concentration and length of exposure. Other compounds, shown to be positive following bath embryo exposure, included additional aflatoxin metabolites and their precursors (e.g., aflatoxicol, aflatoxin G_1, versicolorin A, and sterigmatocystin). Nitrosamines that produced tumors included dimethylnitrosamine (DMN), DEN, nitrosopyrrolidine, and 2,6-dimethylnitrosomorpholine. The direct-acting carcinogen MNNG produced tumors of liver, stomach, kidney, and swim bladder. In general, embryo exposures have led to multi-organ tumorigenesis. Based on the fact that a variety of metabolites and their precursors of aflatoxin resulted in tumor formation several months after trout embryo bath exposure, this is indirect evidence that DNA adduction occurred and that embryos possessed sufficient metabolic machinery to drive the process. Additional evidence for this was provided by a breakthrough in embryo exposures: direct microinjection of the compound (Metcalfe and Sonstegard, 1984). Liver tumors were induced in rainbow trout 1 year after carcinogens were microinjected into embryos. Neoplasms were induced by a single injection of 13 or 25 ng of AFB_1 per egg. Other compounds injected and producing tumors were DMBA and 2-anthramine. Importantly, these investigators demonstrated that over 70% of [^{3}H]-BaP injected into eggs was retained in hatched embryos 120 hours after injection. Exogenous activation of test compounds using rat liver microsomal preparation (S-9) increased the incidence of liver tumors in fish given injections of 25 ng AFB_1. The amount of chemical required for the embryo injection assay was comparable to that required for the Ames bacterial mutagenesis assay. This experiment showed indirectly that, while embryo and hatchling trout are capable of carcinogen bioactivation, additional bioactivation leads to more tumor formation. To date, we have no direct information regarding embryo metabolism of procarcinogens. Although the trout model has provided extensive information on metabolism, it has come from work with larger and older individuals, while most of the initial exposure is taking place in embryos. The possibility exists that we are subjecting information from older individuals down onto embryos.

Other studies contrasting adult vs. early-life-stage exposures involved progression of hepatic neoplasia in medaka following aqueous exposure to DEN (Okihiro and Hinton, 1999). Larvae (2 weeks old) were exposed to 350 or 500 ppm DEN for 48 hours. Adults (3 to 6 months old) were exposed to 50 ppm DEN for 5 weeks. Tumors were markedly different in medaka exposed to DEN as larvae vs. those exposed as adults. Differences included: (1) a higher prevalence of hepatocellular carcinomas in medaka exposed as adults (100% of hepatocellular tumors in adult-exposed medaka were malignant, but only 51.5% of larval hepatocellular tumors were malignant); (2) higher prevalence of biliary tumors in medaka exposed as larvae (46.4% of all tumors in larval-exposed medaka were biliary vs. 8.1% in adult-exposed fish); and (3) higher prevalence of mixed hepatobiliary carcinomas in adult-exposed medaka (24.3%) compared with those exposed as larvae (3%). In addition, a unique hepatocellular lesion termed *nodular proliferation* was only observed in adult-exposed medaka. The lesion was characterized by small size (50 to 300 μm), complete loss of normal tubular architecture, and variable megalocytosis. Nodular proliferation was distinct from preneoplastic foci of cellular alteration and may represent microcarcinomas (Okihiro and Hinton, 1999). It is important to note that the authors cannot ascribe all differences

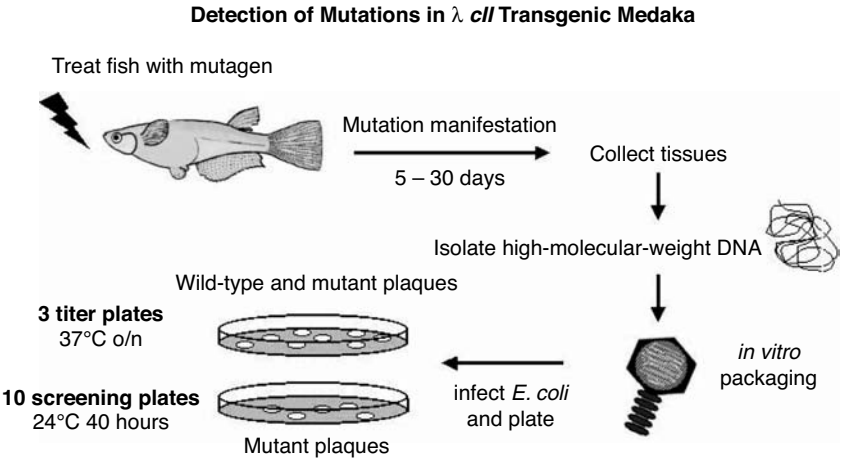


FIGURE 12.9 Detection of mutations in λ *cII* transgenic medaka.

in this study to age at exposure, as exposure conditions were different; however, use of each regime was based on earlier trials in which an appreciable incidence of tumors resulted.

Future Challenges and Opportunities

A 2000 review of human cancer by Hanahan and Weinberg suggested that cancer research will develop "into a logical science where the complexities of the disease, described in the laboratory, … will become understandable in terms of a small number of underlying principles. Some of these principles are even now in the midst of being codified. Our faith in such simplification derives directly from the teachings of cell biology that virtually all mammalian cells carry a similar molecular machinery regulating their proliferation, differentiation, and death. We suggest the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth." The authors of this chapter choose to extend this treatment to include other vertebrates (fishes), or, perhaps more correctly, to point out that the work of our colleagues on fish carcinogenesis over many years serves to reinforce the thesis of Hanahan and Weinberg. We also hypothesize that, through the continued use of fish models, advancements will be made when we also consider the role of epigenetic and other events, such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis during the carcinogenic process following initiation. In this way, new, more acute endpoints for risk assessment may arise, and we will likely learn far more about the mechanism and pathogenesis of chemical-induced neoplasia in fishes.

Bioassay of Chemical Carcinogens

Fishes have played a significant role in our understanding of the potential carcinogenicity of various compounds through the use of a variety of bioassays. In nearly all cases the endpoint is not a tumor; however, assays that effectively measure mutations can in fact illuminate a pathway for furthering our understanding of chemical carcinogenesis to include potential mechanism and the role mutations may play. The interested reader may wish to pursue relevant literature on bacterial mutagenesis, yeast mutagenesis, DNA repair assays, sister chromatid exchange, and gene expression arrays.

One vertebrate fish assay merits further mention here. Along similar lines to the Big Blue® rodent mutation assay (Lambert et al., 2005), transgenic medaka have been developed as a tool to determine the mutation frequency following exposure to environmental mutagens (Figure 12.9) (Shih et al., 2000;

Winn et al., 1995, 2001). Such medaka have been produced containing a prokaryotic vector including a lambda *cII* transgene acting as a mutational target that, in turn, enables quantification of mutation events. Such fish can be treated with a compound, time allowed for mutations to occur, and the genomic DNA isolated to analyze for mutational damage (Winn and Norris, 2005). The many advantages of the assay cited include the ability to screen a large number of copies of a locus rapidly, thus producing statistically reliable information about the frequency of mutations in any selected tissue and requiring fewer animals; the fact that the mutations are fixed, in that they are detected after DNA damage and repair; the genetically neutral status of the target transgene such that it will not be selected against by the animal over time; and, finally, the ability to characterize any mutant to give information about its mechanism of action. Studies of chemical mutagenesis using the transgenic medaka have been conducted and the assay validated. The *cII* target was found to be highly responsive, showing both dose dependence and different mutational spectra following exposure to ENU, DMN, DEN, and BaP (Winn et al., 2000; Winn and Norris, 2005). Compared with controls, the mutation frequencies detected were 7-fold higher in fish exposed to 300 mg/L DEN, 65-fold higher in fish exposed to 100 mg/L DMN, and 5-fold higher in fish exposed to BaP (Winn and Norris, 2005). Such mutation frequencies are similar to those reported using the transgenic Big Blue® rodent mutation assays. While bearing in mind that different exposure routes and durations are involved in rodent mutation frequency assays compared with fish, 2- to 12-fold increases in mutation frequency following BaP and DMN exposures, respectively, have been reported (Shane et al., 1997, 2000a,b). The current transgenic *cII* medaka and any future fish mutagenesis assays will require careful protocol optimization and harmonization, particularly in two specific experimental variables—the administration time and the sampling time—so mutation frequency data can be compared with confidence.

Field Studies

Higher than expected prevalence of hepatic neoplasms has been reported in one or, occasionally, two species of wild fish from each of a total of 28 sites within North America (Harshbarger and Clark, 1990; Vogelbein et al., 1990). When taken together, these epizootics involve 15 different species. In addition, investigations in the North Sea (Kranz and Dethlefsen, 1990) indicate an epizootic of hepatic neoplasms in the pleuronectid, bottom-dwelling dab (*Limanda*). In concluding their review, Harshbarger and Clark (1990) regarded hepatocellular neoplasms of feral fishes to be strongly correlated with exposure of their hosts to chemical contaminants. Risk appeared to be related to life history (Harshbarger and Clark, 1990), and certain bottom-dwelling species were the most commonly affected. Epidermal neoplasms were also found in fish near polluted areas but were regarded as having less of an associative link with chemical carcinogens. Epizootics of hemic, neural, connective tissue, and gonadal neoplasms were interpreted as having the weakest relation to environmental pollution (Harshbarger and Clark, 1990). For these reasons, our attention in this section will focus primarily on hepatic neoplasia. Although we regard neoplasms in other organs to be of importance, the bulk of the field studies and associated laboratory experiments have revolved around this organ. Readers interested in non-liver cancers in other wild populations are referred to a recent review by Ostrander and Rotchell (2005).

It is not surprising that the liver of fishes is a target for toxic chemicals including potentially carcinogenic compounds. This happens because of: (1) its large blood supply, leading to pronounced toxicant exposure and accumulation; (2) its clearance function involving microvasculature, hepatocytes, and possibly phagocytic cells and intrahepatic biliary system; and (3) its pronounced metabolic capacity, critical for internal homeostasis and for survival of the organism. It is this great metabolic capacity of the liver that makes it both a target and an organ to protect itself and the host.

The liver is a major site for biotransformation of potential carcinogens. This has been well characterized in a variety of fishes (Stegeman and Lech, 1991) and involves the cytochrome P450 monooxygenase system, particularly CYP1A (for reviews, see Buhler and Wang-Buhler, 1998; Stegeman and Hahn, 1994). Furthermore, CYP1A shows the highest specific activities in liver (Hahn and Stegeman, 1994) and has been localized (Lorenzana et al., 1989; Smolowitz, et al., 1991) to the cell types (Husoy et al., 1996; Lester et al., 1993; Sarasquete and Segner, 2000) involved in the various degenerative, regenerative, preneoplastic, and neoplastic hepatic lesions (Myers et al., 1987, 1994) associated with environmental

exposure. Due to their well-established role as proven, complete carcinogens in laboratory exposures with rodents (Roe and Waters, 1967) and fishes (Bailey et al., 1987b; Hawkins et al., 1995; Hendricks et al., 1980) and their ubiquitous occurrence in sediments (Gardner et al, 1998; Malins et al., 1985; Myers et al., 1994; Varanasi et al., 1989; Wirgin et al., 1994), with particularly enhanced levels in sediment extracts from highly contaminated sites (Baumann et al., 1996; Malins et al., 1985; Vogelbein et al., 1990), the PAHs have rightfully deserved to be the primary focus of attention in field carcinogenesis studies.

What is the evidence that fish are exposed to these environmental PAHs? This question has been addressed by comparisons of spectra from sediment to those from stomach contents of benthic fishes. Results indicate that PAHs of sediments are also found in analyses of stomach contents of benthic fishes (Myers et al., 1987). Furthermore, a study that involved 27 different sites on the Pacific Coast from Alaska to southern California demonstrated the widespread nature of the contaminant investigations. When exposed, fish readily accumulate PAHs (Varanasi et al., 1987). Although the concentrations of parent compounds in muscle and liver are low, this is now known to reflect prior metabolism by liver. Fluorescent aromatic compounds (FACs) in bile are products of hepatic PAH metabolism, and this provides a means of establishing exposure and comparing uptake in fishes from various sites. FACs analysis has become a widely used approach to demonstrate exposure and host response to PAHs (Myers et al., 1991; Wirgin et al., 1994).

As was covered earlier, the PAHs are procarcinogens and must be metabolized to the ultimate carcinogenic form. Analysis of xenobiotic metabolizing enzymes of English sole from contaminated and reference sites showed differences in levels as a function of the site from which they were collected (Collier and Varanasi, 1991). Furthermore, cohorts held in the lab and fed different diets revealed changes in the enzyme parameters and in FACs in the bile. Varanasi et al. (1989) demonstrated the relevance of the metabolism to carcinogenesis in the English sole. They exposed sole to the parent compound in the laboratory and detected the ultimate carcinogenic form of BaP, the BaP-7,8-diol-9,10 epoxide, and *syn*- as well as *anti*-BaP deoxyguanosine adducts. Subsequently, isolated hepatocyte preparations from sole were used to investigate metabolism of tritium-labeled BaP (Nishimoto et al., 1992). The cells formed conjugated metabolites and *syn*- and *anti*-BaP diol epoxide adducts similar to those formed by intact livers.

What is the evidence that metabolism in wild fish is leading to the formation of adducts? Because adduct formation represents a key step in the initiation of the carcinogenic process, it became important to determine whether fish collected from highly contaminated sites showed more adducts and whether these fish would have greater numbers of adducts when compared to the same parameter in fish collected from reference sites. The study by Varanasi et al. (1989) lent credence to the notion that, of the PAHs, chrysene, BaP, and dibenz(*a*,*h*)anthracene were involved in the adduct formation. Interestingly, they also conducted a comparison between Puget Sound and Boston Harbor in tumor-prone English sole and winter flounder, respectively, and showed similar adduct formation. Stein et al. (1993) investigated the formation and persistence of BaP and 7H-dibenzo(*c*,*g*)carbazole (DBC) adducts. The latter compound, a nitrogen-containing aromatic carcinogenic to rodents but not studied in fish, was important, as it was considered possibly the most significant of the PAHs in sediments. Both compounds formed adducts, and these declined over the 28-day course of study with half-lives of 11 and 13 days, respectively. Further refinements of the ^{32}P post-labeling technique with respect to quantitative analysis of PAH adducts now allows for molecular dosimetry. With carcinogens covalently bound to DNA, an accelerated approach through toxicokinetics to get at the actual delivered dose at the molecular target (Stein et al., 1993) and the endpoints detected are associated with increased exposure to environmental PAHs.

What is the evidence that the PAHs and host biochemical and molecular alterations are related to morphologic alterations in liver, specifically hepatic neoplasms? The evidence comes from statistical analyses. First, when 3 species were studied in the 27 sites on the Pacific coast, all showed significantly higher lesion prevalence in the contaminated urban as opposed to the reference sites (Varanasi et al, 1987). Second, concentrations of PAHs, PCBs, DDT and derivatives, chlordanes, and dieldrin in sediment, stomach contents, and liver and FACs in bile were significant risk factors for the occurrence of hepatic lesions, including lesions designated as neoplastic, preneoplastic, non-neoplastic but proliferative, and degenerative/necrotic lesions, as well as hydropic vacuolation primarily of hepatic biliary epithelium (Myers et al., 1994). Earlier work by this group (Myers et al., 1991) demonstrated significant and consistent statistical associations between levels of aromatic hydrocarbons in sediment and hepatic neoplasms using a logistic regression analysis. Another way to approach this question is to bring the

environmental sample into the laboratory and conduct controlled exposures. This has been done with English sole, winter flounder, and medaka (Gardner et al., 1991; Fabacher et al., 1991; Myers et al., 1991). When sole collected from reference sites were exposed to sediment extracts from contaminated sites, foci of cellular alteration were observed (Myers et al., 1991). When winter flounder (*Pseudopleuronectes americanus*) were fed blue mussels (*Mytilus edulis*) from contaminated Black Rock Harbor, they developed renal and pancreatic neoplasms and hepatic foci of cellular alteration, demonstrating that sediment-bound carcinogens could be trophically transferred to shellfish and to their consumers (Gardner et al., 1991). The Fabacher et al. (1991) study used a surrogate species, medaka, to test the cancer-causing potential of sediment extracts from the Laurentian Great Lakes. Tests with extracts from highly contaminated sites proved positive, and resultant tumors were of the identical organ and cell type as those seen in the field in the species of interest.

When the above studies are viewed in combination, it is clear that the fish developing tumors likely derived their exposure via the diet, although direct contact in these benthic species also remains a possible route. The diagnosis of neoplasms is founded upon well-defined morphologic criteria, and the generation of tumors of the liver involves multiple steps. As such, the findings of overt neoplasms in wild fish coupled with our knowledge of their prior exposure, their appropriate toxicokinetic characteristics to deliver compound to target cells and tissues, their ability to metabolize procarcinogen to an ultimate carcinogenic form, and their ability to form adducts suggest that tumorigenesis in fishes parallels that of rodent models—that is, they experience initiation, promotion, and progression to tumor formation.

Another approach that has proven to be of value is to investigate responders (tumor-prone fish) and compare steps in the carcinogenesis pathway with individuals of closely related species that are more resistant. Two such match-ups include the English sole and starry flounder and the brown bullhead and channel catfish. Reichert and associates (1998) used ^{32}P post-labeling and quantitative determination of resultant adducts to investigate differences between tumor-prone English sole and a resistant cohabitant species, starry flounder. Such comparisons lend strength to the association of contaminants as causal factors in this form of chronic toxicity. Mechanistic differences in toxicokinetics explained differences in dosimetry and reflect some of the reasons related to the differences in prevalence of neoplasia. Previous biochemical investigations by Collier et al. (1992) investigated phase I and phase II enzymes in English sole and starry flounder. Whereas the former showed increased activation and decreased detoxification, the latter had greater potential to detoxify and showed an abundance of isoenzymes of glutathione *S*-transferase. Further, this suggests that the diminished response in starry flounder is not due to any inherent differences in sensitivity to the ultimate carcinogen but to differences in toxicokinetics that diminish adduct formation (i.e., molecular dosimetry) (Reichert et al., 1998). A similar course of investigations has been taken by Di Giulio and his group to analyze responses in brown bullhead, the tumor-prone species in the freshwater studies of the Laurentian Great Lakes and the tidal Potomac watershed in Maryland compared to those of the channel catfish, a closely related species often inhabiting the same sites and one that is resistant to hepatocarcinogenesis. Those investigations have taken a biochemical and a molecular approach as well.

From the above sections of this chapter, a portion of the differences between responders and resistant individuals may arise by as yet uninvestigated factors. These include but are not limited to differential response to promoters (promotional stimuli), differential response to cocarcinogens, differences in host immunosurveillance and tumor cell killing capacity, and possible differences with respect to progressor effects.

The ecological significance of chemically induced neoplasia in fishes has been investigated in few, but fundamentally important, field investigations. The Baumann et al. (1990) study demonstrated that the brown bullheads sampled at the highly contaminated site where tumors were occurring had statistically significant reduction in the contribution to the overall population by older individuals, the 6- to 7-year class. By contrast, population analysis of bullheads from a reference site (Old Woman Creek) revealed considerable contribution (18%) by the 6- to 7-year fish. This has ecological significance, as the response was at the population level. Perhaps the life-shortening nature of carcinogenesis has led to this population-level response. If such mature fish are lost due to early mortality, the number of reproductively active members of the population could be reduced, further diminishing population size.

The bigger question of future population consequences of chemically induced neoplasia has yet to be addressed. Genetic damage that occurs as germ-line mutations has a potentially profound effect for both the individual and population. The development of disease states, including carcinogenesis, usually involves multi-hit events, and mutational damage can be passed to progeny as germ-line mutations. It is therefore possible that the progeny of individuals from contaminated sites may be more susceptible to further mutational damage and subsequent specific disease. In human disease incidence studies, such individuals more prone to disease are referred to as possessing a *mutator phenotype* (Loeb and Loeb, 2000). Individuals with the mutator phenotype are considered subject to an increased susceptibility to further disease. Few studies have investigated the heritability of contaminant-induced DNA damage in fish. One study has identified *ras* gene mutations in directly oil-exposed embryos (Roy et al., 1999) but did not address the question of heritability via parental exposure nor the implications in terms of increased susceptibility for subsequent populations to common environmental contaminants. The detection, induction, and characterization of DNA damage in adult fish and examination of their gametes and offspring for inherited mutational damage could address this question. Thus, establishing the presence of a mutator phenotype in individual fish is an especially novel approach to investigating contaminant-induced carcinogenesis.

Discussion of field investigations must also include the creosote-laden site in the southern branch of the Elizabeth River in Virginia. Important findings there have not only related to an epizootic of hepatic and pancreatic neoplasms in mummichog (*Fundulus heteroclitus*) but have also suggested genotoxic effects that have been expressed at the population level. As such, future studies may reveal interesting information related to cancer sensitivity.

Summary

In our coverage of chemical carcinogenesis of fishes, we have included noteworthy applications of laboratory models to determine mechanistic aspects of carcinogenesis; for example, studies in *Xiphophorus* elucidated the mechanisms converting the tyrosine kinase protooncogene INV-X*mrk* to the oncogene ONC-X*mrk*. We also wish to highlight how the variety of mechanisms of sex determination in fishes can provide interesting model subjects for carcinogenesis and toxicity studies. Some fishes naturally reproduce gynogenetically (Woodhead et al., 1984); in others, due to the plastic nature of their sex determination mechanisms, gynogenesis (Naruse et al., 1985; Sakai et al., 1997; Sarder et al., 1999) or androgenesis (Corley-Smith et al., 1996; Parsons and Thorgaard, 1985) can be induced. By comparison, mouse parthenogenetic embryos die at the early post-implantation stage as a result of developmental requirements for paternally imprinted genes (Strain et al., 1995). Fishes also provide a more practical avenue to produce these genetically homogenous offspring that are valuable tools in genetic, transplant, carcinogenicity, and toxicology studies. Due to their genetic uniformity, the hermaphroditic *Rivulus ocellatus marmoratus* has been used to advantage in hepatocarcinogenesis studies (Goodwin and Grizzle, 1994a,b).

Various fishes demonstrate a high degree of sensitivity to carcinogens in conjunction with short induction periods. This has been shown repeatedly in work with the trout model as reviewed above; for example, hepatomas formed within 3 months and 6 months, respectively, in the teleost *Rivulus marmoratus* exposed to DEN at 400 and 100 ppm for 2 hours (Park and Kim, 1984). Similarly, medaka exposed to 250 ppm diethylnitrosamine for 48 hours developed altered hepatic foci by 12 weeks and hepatic adenomas and carcinomas by 20 weeks after exposure (Teh and Hinton, 1998).

The transparent chorion and resultant ease of observation of development within embryonated eggs of zebrafish and medaka favor their emergence as excellent model organisms for studies of vertebrate genetics, and development and for screening of environmental mutagens (Driever et al., 1996; Haffter et al., 1996; Ishikawa, 2000; Loosli et al., 2000; Shima and Shimada, 1994). Additional positive features are the high fecundity and short generation times that are characteristic of both species and facilitate large-scale genetic screening. In the zebrafish, more than 500 mutant phenotypes have been identified in various aspects of early development, and several specific mutations homologous to human diseases have been described (Wang et al., 1998b).

Zebrafish and medaka, being vertebrates, offer advantages over other large-scale screening models (e.g., *Drosophila* and *Caenorhabditis elegans*) in that they provide more comparative value to human disease investigation. Several examples of clinically relevant zebrafish mutant phenotypes were reviewed, and progress continues to be made in developing molecular and genetic techniques and in building the zebrafish genomic infrastructure (Dooley and Zon, 2000). In medaka, the small genome size (800 Mb) further favors their use as a vertebrate model for genomics, and a large-scale genome-sequencing project has recently been made available at the Japanese National Institute of Genetics (NIG). Additional genomic resources include a medaka radiation hybrid panel, Bac libraries, and ENU mutagenesis screens, among others. These molecular tools have greatly facilitated our ability to engage in complex genetics and genomics research with these models. Hepatic gene expression profiles were examined in trout after dietary exposure compounds shown earlier to be promoters of liver carcinogenesis. These data suggested the mechanisms through which the promoters worked (Tilton et al., 2006). Japanese researchers have developed numerous inbred medaka strains, a process that again is made practical by a short generation interval. These inbred strains are used as tools for genetic research (Hyodo-Taguchi and Egami, 1985; Kubota and Shima, 1991; Ohyama et al., 1986; Wada et al., 1995).

Inbred strains have also been used in radiation biology and carcinogenesis studies (Hyodo-Taguchi and Egami, 1989; Hyodo-Taguchi and Matsudaira, 1987; Nakazawa et al., 1985). Because inbred strains provide more homogenous genetic backgrounds, they offer advantages over outbred strains in control of experimental variability over a broad range of research applications. The use of outbred strains may well reflect the range of responses possible in a heterogeneous population, but the use of inbred strains offers standardization and homogeneity of outcome invaluable for mechanistic studies.

Although the method has not been widely used in the United States, medaka have been successfully cloned using a protocol involving deactivation of sperm that then serve to activate, but not fertilize, eggs from a single female (Naruse et al., 1994). The eggs then begin to divide and develop. This process allows development of identical animals, an ideal method for control of genetic variation and a potential tool for transplant and immunological studies, as well as toxicological and carcinogenesis research. Clearly, the future is bright for laboratory-based studies using fish models; however, this work must not supplant other important aspects of fish in chemical carcinogenesis. Field investigations with our enhanced toolkit must be continued and pursued with greater intensity. Environmental problems are not going away, and observations from organisms within various habitats offer a unique insight into the quality of our planet.

Water covers approximately two thirds of our planet, and, regardless of the initial source of environmental contaminants, they ultimately deposit within the aquatic medium; therefore, sensitive organisms that readily accumulate xenobiotics are likely to be the sources of important information regarding alterations in phenotype and genotype within a changing aquatic environment. For this reason, it is important that mechanisms of toxicity and the acquisition of biological information from laboratory model fishes not become our sole reasons for using these taxa. Because most natural aquatic environments are populated by a variety of fish species, fish models of aquatic toxicity are relevant to broad ecological concerns. Depending on life stage, individual fish may occupy two or more niches during their lifespan. Early life stages of fishes can be exposed to potentially carcinogenic compounds either by maternal exposure through contaminants in yolk or through *trans*-chorionic exposure from the water column or sediment. The growth of fishes is rapid, and if it occurs after initiation such growth may promote the tumorigenic process. The subsequent assumption of a different niche can lead to exposure to substances that may promote the tumorigenic process, and this may arise from a near total change in the food organisms on which the individual is feeding. Such alterations in dietary components in turn may modulate tumor response. Because fishes occupy one or more of a variety of levels in the food chain, frequently dependent on life stage or migration, impacts of contaminants or other physical environment changes can be evaluated at multiple levels using indigenous fishes. It is therefore imperative that communication and cooperation exist between laboratory and field investigators to make use of the information to be gleaned from these unique vertebrates.

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13

Toxicity Resistance

Peter A. Van Veld and Diane E. Nacci

> Fish populations in polluted streams, if not driven to local extinction, may adapt to certain toxic substances over time.
>
> **Angus, 1983**

CONTENTS

Introduction

Chemical Tolerance or Resistance to Toxic Effects

Although chemical contamination of our environment is often associated with human activities, plants and animals have evolved in an environment that has included continuous exposure to toxic materials (Ames et al., 1990a,b; Gribble, 1994; Menzie et al., 1992; Shaw, 1999). Basic mechanisms for resisting toxicity probably evolved with early life and are likely to be highly conserved in nature (Borst, 1993; Doehmer et al., 1993; Doige and Ames, 1993; Gonzales and Nebert, 1990; Gottesman and Pastan, 1993; Hayes and Pulford, 1995; Nebert et al., 1990; Roesjadi, 1992; Sheehan et al., 2001). Because of the large number and wide distribution of novel anthopogenic compounds introduced into the modern world, these mechanisms have become increasingly essential for survival. Organisms surviving environments heavily contaminated with anthropogenic chemicals demonstrate a diversity of mechanisms to tolerate or resist toxic effects.

Resistance or tolerance can be defined as the relative ability to function or survive during toxicant exposures that are harmful or lethal to susceptible individuals and populations. In the laboratory, tolerance is confirmed when chemical exposures that are toxic to individuals from uncontaminated sites are less toxic to individuals with a history of chemical exposure.

Fish and other organisms appear to develop tolerance through a variety of short-term and long-term processes (Benson and Birge, 1985; Chambers and Yarbrough, 1979; Chevillon et al., 1997; Doehmer et al., 1993; Eaton and Bammler, 1989; Fernandez-Salguerro et al., 1996; Kurelec, 1992; Taylor and Feyereisen, 1996; Roesjadi, 1992; Winston, 1991; Winston and Di Giulio, 1991). Physiological acclimation and genetic adaptation are general terms for short-term, transitory responses and long-term, heritable responses, respectively (Table 13.1). Physiological acclimations occur in direct response to toxic exposures and likely involve temporary alterations in the levels of expression of proteins and enzymes involved in chemical defense. Following chemical exposure, protein expression returns to normal and the state of physiological acclimation declines. Genetic adaptation or evolved tolerance occurs when the genetic basis for advantageous responses is passed on to progeny. In genetic adaptation, tolerance is retained through successive generations, even when progeny are not exposed to chemicals (Endler, 1986).

The terms *physiological acclimation* and *genetic adaptation* have been used frequently for categorizing mechanisms of chemical tolerance in fish (Elskus, 2001; Hahn, 1998; Weis and Weis, 1989; Wirgin and Waldman, 1998, 2004); however, other processes and conditions may contribute to tolerance as well (Table 13.1). For example, abundant evidence indicates that some forms of chemically induced cancer

TABLE 13.1

Processes and Conditions Contributing to Resistance

Type of Resistance	Description
Resistance or tolerance	Relative ability of individuals or populations to function or survive during toxicant exposures that is inhibitory or lethal to susceptible (sensitive) individuals or populations of the same species
Physiological acclimation	Resistance based on short-term physiological or biochemical responses to toxicant exposure (e.g., induction of biotransformation enzyme)
Genetic adaptation (evolved tolerance)	Resistance that is passed onto progeny and retained through successive generations; likely involves genetic alterations in target sites, toxicant receptors, and proteins involved in detoxification, toxicant binding, and efflux
Cancer	Resistance resulting from nonheritable gene mutations and biochemical alterations that occur during chemical carcinogenesis
Epigenetic alterations	Resistance resulting from modifications in DNA (e.g., hypermethylation) that do not involve mutations in nucleotide sequence but alters (e.g., silences) gene expression
Nongenetic, heritable tolerance	Resistance resulting from maternal transfer of toxicant or mRNA from parent to offspring; resistance does not likely last beyond one generation

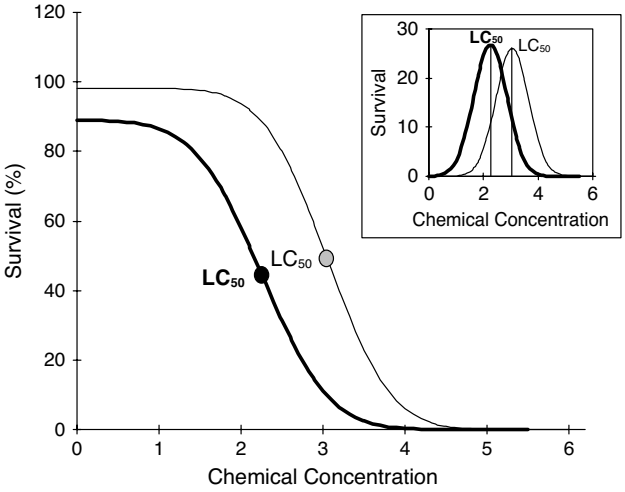

FIGURE 13.1 Cumulative and noncumulative (inset) distributions indicating population responses to chemical exposures. Lethal response curves and 50% lethal effect concentrations (LC_{50}) are shown for a sensitive population (bold curve) and a more tolerant (light curve) population.

represent adaptations to harsh chemical environments, providing survival value to individuals especially during the early stages of cancer (Farber and Rubin, 1991). Cancer resulting from chemically induced mutations in somatic cells and concomitant alterations in protein expression would be considered to be a genetic but nonheritable adaptation. In addition to cancer, epigenetic alterations, such as hypermethylation of promotor regions of DNA, may affect responsiveness to drug and chemical exposures. Although gene silencing due to hypermethylation has been widely studied in mammalian cancer research (Goodman and Watson, 2002), it has only recently being investigated as a tolerance mechanism in fish (Arzuaga et al., 2004; Timme-Laragy et al., 2005). Finally, nongenetic but heritable factors involving maternal transfer of toxicant from an exposed parent to offspring could contribute to tolerance in offspring. In cases involving maternal transfer, tolerance may appear to have a genetic basis (i.e., tolerant field-collected parents and their progeny) but is in fact physiologically based and related to direct exposure of offspring to toxicant. Because of the possibility of maternal transfer, genetic adaptation is established only when tolerance is maintained for two or more generations.

In populations inhabiting severely contaminated sites, multiple processes likely contribute to resistance during the lives of individuals; for example, physiological acclimations could provide individuals with the ability to survive and reproduce in moderately contaminated sites. As chemical contamination at a particular site increases with time, selection of progeny that carry genetic traits with adaptive significance could become a more dominant factor. Genetically adapted individuals may rely to some extent on epigenetic mechanisms or may respond to periodic pulses of contaminants through physiological responses. Tumor-bearing individuals may also exhibit features characteristic of genetic adaptation, physiological acclimation, or epigenetic alteration.

Variation in Chemical Response

Sensitivity to toxicants is often determined by conducting standardized bioassays and expressing results as the chemical concentration producing an adverse biological effect in some proportion of the tested group (e.g., fish species or population). The LC_{50}, or mean lethal concentration, is the chemical concentration killing 50% of the tested group (Figure 13.1). LC_{50} values provide a convenient way of comparing or ranking sensitivities of tested groups. Lethal concentrations are high (or shifted right) in tolerance relative to more sensitive groups (Figure 13.1); however, all individuals within tested groups do not respond identically, and even within sensitive groups some individuals are more tolerant than others (Figure 13.1 inset).

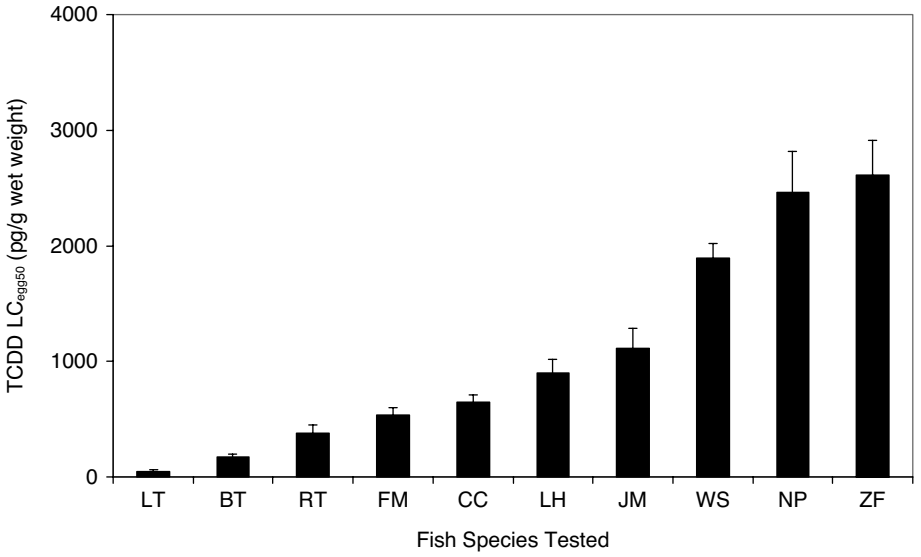

FIGURE 13.2 Variation in response to TCDD (LC_{50} and 95% confidence intervals) in early life stages of lake trout (LT), brook trout (BT), rainbow trout (RT), fathead minnow (FM), channel catfish (CC), lake herring (LH), Japanese medaka (JM), white sucker (WS), northern pike (NP), and zebrafish (ZF). (From Elonen, G.E. et al., *Environ. Toxicol. Chem.*, 17(3), 472–483, 1998. With permission.)

Different species of fish can vary dramatically in their sensitivity to specific chemicals; for example, the LC_{50} values for the early life stages of eight species of freshwater fish for the potent organic contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) vary by over 100-fold (Figure 13.2). This means that dioxin concentrations that kill a large proportion (50%) of exposed individuals from a sensitive fish species would kill very few (but perhaps some) individuals from a more tolerant fish species. Considered in this way, differences in sensitivities between fish species or populations reflect changes in the proportion of sensitive individuals within the group (as in Figure 13.1 inset).

Within a single fish species, the sensitivities of different populations can be affected by prior chemical exposures. By definition, individuals surviving toxic exposures are relatively tolerant, and where tolerance has a genetic basis those tolerant survivors will produce tolerant progeny. In this manner, toxic exposures over multiple generations can produce chemically adapted fish populations that contain relatively high proportions of tolerant individuals and demonstrate increased (right-shifted) mean lethal concentrations relative to the original or unexposed population (Figure 13.3). Large variation in tolerance among fish populations within a single fish species provides evidence that this tolerance has some genetic basis, and other outcomes related to these genetic changes (not all beneficial) are considered later in this chapter.

Factors Influencing the Ability of Populations to Become Tolerant

Numerous factors related to the type of contaminant, contaminant exposure, and features of the exposed species can contribute to the likelihood and effectiveness of toxicity resistance in fish populations. Different categories of toxic chemicals affect fish by different mechanisms and require different mechanisms of adaptation. The toxic potency of the specific chemicals involved as well as the frequency, duration, and magnitude of exposure also contribute to tolerance. Persistent contaminants can produce long-term exposures, sometimes generations after discharge has ceased. Even nonpersistent chemicals can produce persistent exposure when discharge continues over time. Species characteristics related to life history (e.g., diet, behavior, reproduction, migration) and demography (e.g., population size, genetic diversity) contribute to exposure and the ability to tolerate exposure. Although different species vary in their inherent chemical sensitivities (Figure 13.1), tolerance is most often observed in nonmigratory species inhabiting contaminated sites through multiple generations. Tolerance appears to develop in

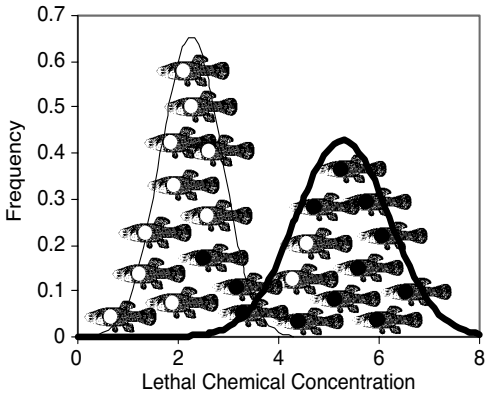

FIGURE 13.3 Hypothetical shift in distributions of sensitive (white spot) and tolerant (black spot) genotypes in fish population following selection by a chemical stressor.

generalists or opportunists that are broadly resilient to a wide range of environmental conditions (e.g., temperature, dissolved oxygen, salinity). In addition, those species most suited for genetic adaptation are likely to have (1) short generation times, (2) large population numbers, and (3) high genetic diversity.

Environmental Contaminants That Produce Tolerance in Fish

Chemical resistance in fish has been observed in response to diverse environmental contaminants, including pesticides, dioxin-like compounds, polycyclic aromatic hydrocarbons (and other compounds associated with creosote), and metals (Table 13.2). A brief description of the occurrence, use, and production of these contaminants provides a better understanding of the relationships among human activities, contaminant exposures, and the development of tolerance in specific fish species and populations.

Organochlorine Pesticides

Dichlorodiphenyltrichloroethane (DDT), the first highly effective, modern organochlorine pesticide, came into widespread use shortly after World War II (Hites and Eisenreich, 1987; Laws, 1993; Manahan, 1999; Plumbic, 2001; Ware, 1991). Early results indicated exceptional performance in diverse agriculture, forestry, and human health-related applications. DDT killed insects on contact and continued to kill for weeks and months. Slowness to decompose (persistence) was viewed as desirable because DDT was believed to be toxic to insects but safe for humans and other vertebrates. Reappearance of pests (development of resistance) was first believed to mean that not enough DDT had been applied; however, by the late 1950s and early 1960s, it was becoming apparent that because of development of chemical resistance in insects, DDT failed to remain effective. At about the same time, we learned that DDT was harmful to all animals, including vertebrates. It accumulated in food chains and was distributed globally via atmospheric transport and other processes. In 1972, when DDT was banned in the United States, other organochlorine pesticides, structurally similar to DDT, were either in production or were produced shortly thereafter. These include chlordane, aldrin, dieldrin, heptachlor, toxaphene, and chlordecone (Kepone), all of which were subsequently banned in the United States because of problems similar to those associated with DDT. Many of these pesticides are in use today in other countries. DDT and other organochlorine pesticides share properties of poor water solubility, high lipid solubility, slowness to decompose, and great potential for bioaccumulation. Organochlorine pesticides exhibit a broad range of well-documented toxicities. They are best known as potent neurotoxicants, acting on various neuroreceptors and ion channels (ffrench-Constant et al., 1998) and as environmental estrogens that influence reproductive function via numerous pathways (Chedrese and Feyles, 2001; Lundholm, 1997; Rosselli et al., 2000). Modern pesticides in use today, including organophosphate insecticides, pyrethroids, and carbamates, are generally less persistent and more selectively toxic than organochlorine pesticides.

TABLE 13.2

Classes and Sources of Environmental Toxicants Addressed in Toxicity Resistance Studies

Toxicant	Source
Organochlorine pesticides	Includes DDT, the first modern highly toxic pesticide, followed by toxaphene, chlordane, aldrin, dieldrin, heptachlor, mirex, and Kepone. Very persistent compounds that accumulate in fatty tissues and sediments, they are toxic to fish, wildlife, and humans and are banned in the United States. Some of the earliest records of toxicity resistance involve DDT.
Dioxin-like compounds (DLCs)	Includes polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (PCDDs), and other persistent polyhalogenated aromatic hydrocarbons (PHAHs). PCBs were valuable industrial materials used in capacitors, transformers, and other products. PCDDs are produced inadvertently during a variety of processes (e.g., pesticide manufacture, chlorine bleaching of pulp). The most notorious DLC is 2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD), often referred to simply as dioxin.
Polycyclic aromatic hydrocarbons (PAHs)	Complex mixtures of compounds produced during combustion of organic materials, especially fossil fuels; also, natural components of petroleum. PAHs are widely studied because of their abundance in the environment and because of the mutagenic and carcinogenic properties of some members (e.g., benzo(*a*)pyrene).
Creosote	Abundant pesticide mixture used to protect wood pilings, telephone poles, etc., from microbial decay. Creosote is composed primarily of PAHs; nitrogen-, sulfur-, and oxygen- heterocyclic compounds; and phenols.
Metals	Naturally occurring elements (e.g., mercury, lead, cadmium, chromium), including some biologically essential elements (copper, iron, zinc). Human activities alter the environmental loading, availability, and toxicity of metals through a variety of activities such as strip mining, fossil fuel combustion, smelting, and industrial processes.

Dioxin-Like Compounds

Some of the most persistent environmental contaminants are members of a large class of compounds referred to as *polyhalogenated aromatic hydrocarbons* (PHAHs) (Eisler and Belisle, 1996; Fletcher and Mckay, 1993; Rappe, 1991) (see Chapter 5). In the 1960s, during extensive investigations of chlorinated pesticides in fish and wildlife, chemical analysis often revealed the presence of similar, although unidentified, compounds in environmental samples. These compounds, later identified as polychlorinated biphenyls (PCBs), are among the most abundant and widespread contaminants in our environment. PCBs are mixtures of synthetic industrial compounds that were produced for use as plasticizers, dielectric fluids in capacitors and transformers, flame retardants, and many other purposes. Properties that made PCBs valuable industrial materials are also responsible for their persistence in the environment. Like organochlorine pesticides, PCBs are poorly soluble in water, highly soluble in lipid, resistant to degradation, and highly bioaccumulative. Atmospheric transport has resulted in redistribution of PCBs to remote world locations. Production of PCBs in the United States and Europe was banned in the 1970s. Because of their abundance, persistence, and widespread global distribution, they will continue to be important contaminants of concern for decades. It is also possible that PCB mixtures are still in use today in some countries. Approximately 102 PCB congeners, or chemical forms, exist in the environment. Congeners are identified by position and number of chlorine atoms and by the spatial orientation (coplanar vs. noncoplanar) of the phenyl rings. Most toxicological research focuses on coplanar congeners whose toxicological potency exceeds that of noncoplanar congeners. The mode of toxicity of coplanar PCBs allows them to be grouped within a broad, mechanistic-based class of environmental contaminants referred to as *dioxin-like compounds* (DLCs). DLCs are named for their toxicological similarity to TCDD, the most potent member of the DLC family (Safe, 1986). The DLC family includes coplanar PCBs, polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). Although PCBs

were manufactured intentionally, PCDDs and PCDFs can be produced inadvertently during a variety of processes, including wood pulp bleaching, pesticide manufacture, combustion processes, and the manufacture of PCBs. The chemical and physical properties of these DLCs as well as their environmental behavior are similar to those of PCBs.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), produced during combustion of fossil fuels in industry, internal combustion engines, and home heating, are the most abundant toxicants in the marine environment (Manahan, 1999). They are also important components of petroleum and can enter the marine environment through marine transportation activities, accidental spills, improper disposal of used crankcase oil, urban runoff, and numerous other routes, including creosote use and spills (Huggett et al., 1992; Huntley et al., 1995; O'Connor and Huggett, 1988). In the environment, PAHs exist as complex mixtures of many individual compounds that differ primarily by number and position of aromatic rings. In addition, petroleum-derived PAH mixtures exhibit a variety of alkyl-substituted forms. Like DLCs, PAHs generally exhibit low water solubility, accumulate to high levels in sediment, and are readily taken up by aquatic organisms. Although PAHs are more susceptible to microbial degradation and photodegradation than DLCs, they can exist for decades in sediment, particularly under anoxic conditions. In addition, PAHs are continuously introduced into the aquatic environment through fossil-fuel combustion, particularly in urban areas. Although exhibiting relatively low acute toxicity, they are widely studied because of their abundance and because of a relationship between PAH exposure and cancer in vertebrates, including fish (Huggett et al., 1992; Johnson and Landahl, 1994; Varanasi, 1989).

Creosote

Creosote is a pesticide derived from coal tar that is used to protect railroad ties, marine pilings, telephone poles, and other wooden structures against microbial (e.g., fungal) decay and marine fouling organisms (Johansen et al., 1997; Mueller et al., 1989). On a volume basis, it is the probably the most abundant pesticide in the environment. Creosote is a complex mixtures of chemicals composed of approximately 85% PAHs, 10% phenols, and 5% nitrogen-, sulfur-, and oxygen-containing heterocyclic compounds (Mueller et al., 1989). It is often amended with pentachlorophenol to enhance its effectiveness as an antimicrobial agent. Individual compounds found in creosote are susceptible to various degrees of biotic and abiotic degradation processes; thus, although creosote is considered to be highly persistent, measurable losses of the more labile components occur with time. Most of the acute toxicity associated with creosote exposure can be attributed to the heterocyclic components and phenols.

Metals

Metals enter the environment though multiple sources and often exist in contaminated industrial sites with complex mixtures of organic contaminants (Forstner and Wittman, 1981; Furness and Rainbow, 1990; Helz and Huggett, 1987; Newman and McIntosh, 1991). Although metals are naturally occurring and in many cases play essential roles in life, human-related activities increase the entry, bioavailability, and toxicity of metals. Examples of activities and processes that increase the entry of metals (e.g., cadmium, copper, zinc, lead) into the environment include strip mining and mine runoff, runoff from fly ash piles, smelting, and combustion of fossil fuel. Numerous human-related activities can increase the bioavailability of metals as well; for example, excessive nutrient use often enhances formation of anaerobic sediments, which in turn enhances conversion of mercury to highly bioavailable and toxic methylmercury. Other industrial processes increase the toxicity of metals by altering their speciation; for example, the manufacture of chromium products often involves conversion of trivalent chromium to hexavalent chromium, the most biologically active and toxic form. Metal speciation and bioavailability are also altered by diverse environmental conditions (e.g., pH, salinity, water hardness, sediment chemistry, dissolved organics), all of which can have large effects on metal toxicity (Roesijadi and Robinson, 1994).

Biochemical Mechanisms and Models That Contribute to Our Understanding of Tolerance

Biological resistance to drugs and toxic chemicals has been studied in diverse organisms because of issues relating to drug resistance in the medical field (e.g., cancer, infectious diseases) and issues related to insecticide resistance in the fields of agriculture, forestry, and public health. To our knowledge, direct cause (molecular alterations) and effect (development of resistance) relationships have not been established in the less widely studied fish systems; however, similarities likely exist in mechanisms underlying resistance across phyla (Bunger et al., 2003; Daborn et al., 2002; Doehmer et al., 1993; Gottesman, 2002; Oakeshott et al., 2003; Shaw, 1999; Taylor and Feyereisen, 1996). Studies in the more highly explored mammalian and insect systems have provided the basis for hypothesizing and testing associations between molecular responses and chemical tolerance in fish. The toxicity of all chemicals (drugs and toxicants) is dependent on their interaction with cell targets and receptors. General mechanisms by which tolerance is achieved reflect alterations in these targets or receptors or activation of pathways that reduce the amount of chemical reaching the target/receptor (Table 13.3). Modification of a toxicity target can result in reduced damage to the target by the chemical. DDT resistance in insects appears in some cases to involve modification of ion channels that represent toxicity targets for this pesticide. Tolerance might also be achieved by modification of a cellular receptor that normally recognizes and interacts with the chemical, triggering a signal transduction cascade that leads to a toxic response. In this case, modification of the receptor can result in reduced toxicant-receptor interaction, producing a concomitant reduction in toxicity. Alterations in one or more of the signal transduction components that become activated by toxicant–receptor interaction could also contribute to tolerance. Resistance can also result from upregulation (induction) of proteins involved in toxicant sequestration or binding. Other commonly studied resistance mechanisms involve altered regulation of proteins and enzymes involved in toxicant biotransformation (detoxification or activation) or efflux (elimination). Several widely studied molecular responses addressed in studies of pollution-tolerant fish fall into the framework provided in Table 13.3. Examples of underlying mechanisms that have been addressed in resistant fish include altered toxicant–receptor interaction (e.g., aryl hydrocarbon receptor and related signal transduction pathway), sequestration (e.g., metallothionein), biotransformation (e.g., cytochrome P450, glutathione transferases), and efflux (e.g., P-glycoprotein). A brief overview of these systems will provide a better understanding of the role they might play in toxicity resistance.

Aryl Hydrocarbon Receptor

Most of the toxic effects of DLCs in vertebrates are believed to be mediated by their binding to the aryl hydrocarbon receptor (AhR) and activation of the AhR signal transduction pathway (Birnbaum, 1994; Bunger et al., 2003; Kirkvliet et al., 1995; Thurmond et al., 1999) (see Chapter 5). AhR signaling and toxicity involve passage of the DLC–AhR complex into the nucleus (Hahn, 2001; Karchner et al., 2002; Mimura et al., 1999). In the nucleus, DLC–AhR becomes associated with the AhR nuclear translocator (ARNT) to form a DLC–AhR–ARNT complex. This complex has a high affinity for xenobiotic responsive elements (XREs) located in the promotor region of DLC responsive genes. AhR-mediated toxicities are believed to result from altered or inappropriate transcriptional regulation of proteins involved in cell growth, regulation, development, and immune function (Birnbaum, 1994; Kerkvliet, 1995, Toomey et al., 2001; Van den Heuvel and Lucier, 1993).

Strong evidence indicates a close relationship between DLC–AhR strength of binding and degree of toxicity of individual DLCs; for example, the most toxic DLC known, TCDD, binds with higher affinity than other DLCs. PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) and PCB 77 (3,3′,4,4′-tetrachlorobiphenyl) are among the most potent AhR agonists and most toxic PCB congeners. Invertebrates lacking an AhR that binds DLCs (Hahn, 1998) or strains of mice that carry a defective AhR (Fernandez-Salguero et al., 1996) are relatively insensitive to DLCs. Two forms of AhR (AhR1 and AhR2) have been identified in some fish species, including a species for which DLC-adapted populations have been identified, *Fundulus heteroclitus* (Hahn et al., 2000).

TABLE 13.3

General Mechanisms Addressed in Studies of Toxicity Resistance

Mechanism	Description
Toxicity target or receptor	Upon entering a cell a toxicant must interact with a cellular target or receptor in order to exert effects; for example, binding of dioxin-like compounds (DLCs) with the aryl hydrocarbon receptor (AhR) mediates the toxic effects of DLCs. Alterations (e.g., polymorphisms) in the AhR could alter toxicity via altered strength of binding or other mechanisms.
Biotransformation	The fate and effect of many toxicants are governed by their susceptibility to enzymatic biotransformation. Biotransformation by phase I enzymes (e.g., cytochromes P450) and phase II enzymes (e.g., glutathione *S*-transferases) generally yields products that are less toxic and easier to eliminate. Examples of activation (i.e., increased toxicity) of relatively harmless compounds exist, however. Differences in expression of these enzymes have a large effect on cell concentrations of toxicants and therefore on toxicity.
Sequestration	Proteins and other molecules in the cell can reduce availability of toxicants to target sites and receptors through toxicant sequestration or binding. In particular, metallothioneins are a family of metal-binding proteins involved in reducing metal toxicity. Upregulation of metallothioneins is often observed in metal-tolerant fish.
Elimination	Proteins such as P-glycoprotein (Pgp) residing on the plasma membrane of cells are involved in ATP-driven efflux of diverse drugs and toxicants from the cell. One form of Pgp, the multidrug-resistant protein (Mdr), is a major obstacle to treatment of cancer because of its efficiency in pumping cancer drugs out of the cell. P-glycoprotein expression has been reported in a number of aquatic organisms, including fish.

Cytochrome P450

The fate and effects of many drugs and toxicants are governed by their susceptibility to enzymatic biotransformation (Di Guilio et al., 1995; Stegeman et al., 1992) (see Chapter 4). Biotransformation is often a sequence of events involving phase I and phase II reactions. In phase I reactions, a polar group, such as a hydroxyl group, is introduced into the molecule. In many cases, hydroxylation is preceded by an epoxide intermediate. Phase I reactions are catalyzed primarily by cytochrome P450 (CYP)-mediated monooxygenase (MO) systems (Di Guilio et al., 1995; Stegeman and Hahn, 1994). Biotransformation usually results in products that are less toxic and easier to eliminate than parent compounds; however, numerous examples exist in which relatively harmless compounds are transformed by specific reactions to yield cytotoxic and genotoxic products. Perhaps the best characterized example of activation is the biotransformation of a common environmental PAH, benzo(*a*)pyrene (BaP), into cytotoxic and mutagenic BaP diol epoxides (Baird and Ralston, 1997). Exposure to various environmental toxicants and drugs results in elevation or induction of specific CYP forms and concomitant increases in rates of CYP-mediated catalytic activity (Buchneli and Fent, 1995). CYP1A is the major CYP form induced by exposure to specific PAHs and DLCs in fish (Stegeman and Hahn, 1994). CYP1A levels are usually estimated via immunodetection or by measurement of ethoxyresorufin-*O*-deethylase (EROD), a CYP1A-dependent activity. CYP1A induction is the most sensitive and best characterized response to both DLC and PAH exposure and is often used as a biomarker of exposure to both classes of compounds (Whyte et al., 2000). AhR-mediated induction of CYP1A by both PAHs and DLCs suggests a common mechanism of toxic action for both classes of compounds; however, although specific PAHs (e.g., BaP) and DLCs (e.g., TCDD) bind to the AhR and induce CYP1A, large differences exist in the cellular behavior and mechanisms of toxicity for PAHs and DLCs. The toxicity of PAHs primarily involves CYP1A-mediated activation to cytotoxic and genotoxic products (Uno et al., 2001). In contrast, the toxic effects of DLCs involve changes in gene expression mediated by DLC–AhR interactions. In either case, the relative induction of CYP1A serves as an indicator of the response of the organism to incoming DLCs and PAHs. Induction of CYP1A by either DLCs or PAHs may induce toxicity via generation of reactive oxygen species produced during inefficient use of oxygen during CYP1A-mediated biotransformation of xenobiotics (Schlezinger et al., 1999) (see Chapter 4 and Chapter 5). PAHs are sometimes activated by ultraviolet light (photoactivated) into toxic products via non-CYP1A-mediated pathways.

Glutathione *S*-Transferases and Other Phase II Enzymes

Compounds with polar side groups or compounds that acquire polar groups during phase I (i.e., CYP-mediated) biotransformation are susceptible to phase II reactions (Di Guilio et al., 1995; Stegeman et al., 1992) (see Chapter 4). Phase II enzymes use the polar group as a handle to link the compound to various polar endogenous compounds (sugar derivatives, amino acids, peptides, sulfate). These reactions serve to reduce toxicity and to enhance water solubility and elimination. Among the phase II enzyme systems, the glutathione *S*-transferases (GSTs) are a particularly versatile, multifunctional family of detoxification enzymes (Eaton and Bammler, 1999; Hayes and Pulford, 1995; Mannervik et al., 1985; Salinas and Wong, 1999). Reactions involving conjugation of epoxide-containing compounds to glutathione reduce the likelihood of cytotoxicity and genotoxicity resulting from electrophilic attack of epoxide groups on DNA and other cellular macromolecules (Eriksson and Andersson, 1992; Ito et al., 1988). Numerous products of oxidative stress, resulting from phase I reactions and lipid peroxidation, are GST substrates as well (Hayes and Strange, 1995). Furthermore, most GSTs are soluble proteins and can sometimes participate in the transport of lipophilic toxicants to phase I enzymes, thereby enhancing phase I reactions. In addition, GSTs play a sacrificial role by covalently binding to activated phase I products, reducing the likelihood that these products will bind to and damage other cellular macromolecules.

P-Glycoprotein

P-glycoproteins (Pgps) are membrane proteins involved in ATP-dependent efflux of diverse cytotoxic drugs from many drug-resistant mammalian cell lines and drug-resistant tumors (Ambudkar et. al, 1999; Arceci, 1993; Gottesman and Pastan, 1993). Pgps have been identified in a variety of normal tissue and cell types, including the plasma membrane of epithelial cells in the kidney, intestine, and liver (Thiebaut et al., 1989), as well as the endothelia of the blood–organ barriers in testes and brain (Cordon-Cardo et al., 1990; Thiebaut et al., 1989). These sites of expression, along with the role of Pgp in conferring drug resistance, suggest that Pgps may be involved in defense against toxic compounds (Albertus and Laine, 2001; Bain et al., 1997). Mice lacking one of the Pgp genes (*mdr1a*) exhibit decreased elimination, increased accumulation, and enhanced sensitivity to drugs (Schinkel et al., 1994). The ability of pollution-tolerant organisms to simultaneously cope with mixtures of diverse toxic compounds is reminiscent of the broad spectrum resistance to xenobiotics observed in multidrug-resistant mammalian cells. Numerous investigators have speculated that the activity of Pgps may contribute to pollution tolerance in populations of aquatic species (Kurelec, 1992). In support of this hypothesis, Pgp-like proteins have been detected in excretory tissues of several pollution or toxin tolerant aquatic invertebrates (Cornwall et al., 1995; Galgani et al., 1996; Kurelec, 1992; Toomey and Epel, 1993). There is also evidence that Pgp expression and activity in aquatic invertebrates may be elevated by exposure to toxicants (Kurelec et al., 1995, 1996; Minier et al., 1993), although the mechanism of elevation unknown. The majority of work has focused on aquatic invertebrates, but Pgp expression has also been studied in fish, including populations inhabiting contaminated environments (Bard, 2000; Bard et al., 2002a,b; Cooper et. al., 1996, 1999a,b; Vogelbein et al., 1999).

Metallothionein

Metallothioneins (MTs) are a family of metal-binding proteins whose synthesis can be induced by exposure to many metals, including cadmium, copper, zinc, mercury, and nickel (Roesijadi, 1992, 1996; Roesjadi and Robinson, 1994). MTs are believed to play a role in metal regulation and detoxification in diverse microorganisms, plants, and animals (Leber and Miya, 1976; Shaw, 1999). Studies with mutant yeast containing a defective MT provide the most direct evidence for involvement of MTs in protection against metals (Hamer et al., 1985). These yeast function normally but are unable to survive doses of copper that are tolerated by normal yeast. In other studies, MT in the larvae of various metal-sensitive mayfly species was found to be unresponsive to induction by metal exposure (Aoki et al., 1989). Other studies indicate an association between increased metal tolerance and MT induction in fish and other aquatic organisms (Bradley and Sprague, 1985; Bryan, 1976; Dixon and Sprague, 1981a,b,c; Klerks and Bartholomew, 1991; McCarter and Roch, 1983; Stegeman et al., 1992).

Antioxidant Defense

Reactive oxygen species (ROS), including hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide, are produced through a variety of mechanisms during normal cell function (Halliwell and Gutteridge, 1985; Mates, 2000; Winston, 1991; Winston and Di Giulio, 1991) (see Chapter 6). Exposures to several various classes of environmental contaminants are known to enhance the production of ROS through a variety of mechanisms (Di Guilio et al., 1995; Hassoun and Stohs, 1996; Kelly et al., 1998); for example, inefficient use of oxygen and electron transfer during CYP-mediated biotransformation reactions results in formation of ROS. Quinone metabolites produced during CYP-mediated biotransformation of PAHs and other chemicals also contribute to the formation of ROS. Cells contain a number of antioxidant defense mechanisms to protect themselves from potentially harmful effects of these unstable compounds. These defenses include a variety of enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) and small molecules, such as ascorbic acid and glutathione, involved in either ROS scavenging or transformation of ROS into harmless products. A variety of ROS-mediated toxicities, including lipid peroxidation of cell membranes and oxidative damage to proteins and DNA, can result when chemical exposures overwhelm antioxidant defense mechanisms.

Cancer as an Adaptive Response

Much work on the adaptive significance of cancer has involved investigations of chemically induced cancer and drug resistance in humans and rodent cell lines (Gottesman, 2002). Many cancer drugs are cytotoxic; they are designed to kill specific cells and can be considered toxicants. The development of certain forms of environmentally induced cancers (e.g., hepatocellular carcinoma) has been viewed as an adaptation to chemical exposure (Bok, 1989; Eriksson and Andersson, 1992; Farber, 1990; Farber and Rubin, 1991; Monceviciute-Eringiene, 1996; Rubin et al., 1995; Solt and Farber, 1976). Cancer is a disease that develops over one half to two thirds of an organism's life. Only in the later stages do tumor cells acquire properties of autonomy and invasiveness that ultimately can lead to an individual's death. In the earlier stages of cancer, molecular and biochemical changes within developing nodules or preneoplastic lesions can confer chemical resistance to the individual.

The resistant hepatocyte model of chemical carcinogenesis is based on the hypothesis that within the liver there exist rare hepatocytes that upon exposure to chemical carcinogens undergo alterations through carcinogen-induced mutations (Farber, 1990; Gindi et al., 1994; Yusuf et al., 1999). The initiated rare hepatocytes acquire through these mutations a battery of mechanisms that allow them to resist, survive, and proliferate during exposure to doses of toxicants that inhibit or kill adjacent normal cells. In this environment, rare hepatocytes gain a growth advantage over normal tissues and form regions of focal proliferations (hepatocyte nodules) that essentially represent a new liver (Figure 13.4). The nodules, through their resistance to diverse cytotoxic agents, confer protection from acute cytotoxic exposure not only to the liver but also in the whole organism. In support of this theory, rodents bearing aflatoxin-induced hepatic nodules became resistant to doses of carbon tetrachloride that killed 100% of non-tumor-bearing individuals (Harris et al., 1989).

Alterations contributing to drug and resistance often involve proteins and enzymes involved in drug biotransformation and efflux, especially CYP proteins, GSTs, and Pgp (Buchmann et al., 1985; Clynes, 1994; Roomi et al., 1985). Cellular adaptations during carcinogenesis often result in decreased expression of CYP proteins, resulting in decreased transformation of drugs to their therapeutically active (e.g., cytotoxic) forms. This adaptation also helps targeted cells evade the toxic effects of the drug designed to inhibit or kill them. In one study, resistance of human breast epithelial breast cancer cells to PAH-induced cell death (apoptosis) was found to be the result of reduced expression of AhR and CYP1A (Ciolino et al., 2002). In organisms inhabiting severely contaminated sites, decreased levels and activity of CYP proteins could result in decreased activation of toxicants to cytotoxic and mutagenic metabolites. In addition, ROS production by CYP proteins would be reduced in cells that underexpress these enzymes.

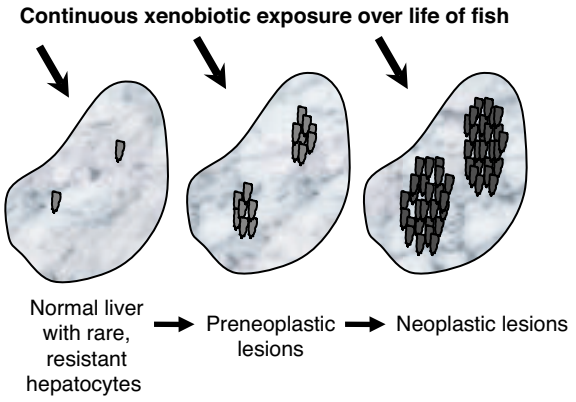

FIGURE 13.4 Resistant hepatocyte model of chemical resistance. Continuous xenobiotic exposure leads to selection for, and proliferation of, resistant hepatocytes featuring depressed CYP, elevated GSTs, and elevated P-glycoprotein. The overall effect would be reduced toxicant activation, elevated detoxification, and elevated efflux.

Another common characteristic of drug-resistant neoplastic cells is overexpression of GSTs and other phase II enzymes (Buchmann et al., 1985; Clynes, 1994; Eriksson and Andersson, 1992; Hayes and Pulford, 1995; Ito et al., 1988). Upregulation of phase II enzymes in cancer therapy could result in increased deactivation and elimination of cancer drugs, decreasing the effectiveness of treatment. In organisms inhabiting contaminated sites, GSTs and other phase II enzymes likely play a major role in protection from toxic effects as well.

A third feature of cancer cells and tissues is overexpression of Pgp and related proteins involved in drug efflux. In many forms of human cancer, Pgp is believed to be the dominant obstacle to cancer therapy. Together with decreased drug activation by CYP proteins and enhanced detoxification by phase II enzymes, elevated Pgp can greatly reduce the effective dose of a variety of drugs by increasing the efficiency of efflux by target cells (Gottesman, 2002). Patterns of protein expression observed in cells of tumor-bearing populations of fish appear similar to the mammalian cancer-drug-resistant phenotype (Armknecht et al., 1998; Cooper et al., 1999a,b; Hayes et al., 1990; Kirby et al., 1990; Van Veld and Westbrook, 1995; Van Veld et al., 1991, 1992) and will be discussed more thoroughly below.

Case Studies: Chemical-Specific Tolerance

Studies addressing chemical tolerance in fish have often focused on populations inhabiting sites severely contaminated with specific compounds. Organized by chemical class, these case studies are discussed to illustrate how tolerance has been demonstrated, and those biochemical mechanisms believed to contribute to chemical resistance in fish populations.

Tolerance to Organochlorine Pesticides

In the 1950s and 1960s, little was known about the fate and effects of organochlorine pesticides or toxicity resistance in fish and wildlife. Resistance to DDT was initially viewed as a curious agricultural problem restricted to insects (Brown, 1958). Later, Ozburn and Morrison (1962) reported a DDT-resistant strain of laboratory mouse, selected by breeding survivors of DDT exposures. These mice retained tolerance into the ninth generation and provided the first evidence of genetic adaptation to pesticides in vertebrates. In 1963, the first report of pesticide resistance in natural populations of vertebrates involved DDT resistance in frogs (Boyd et al., 1963). In that study, cricket frogs (*Acris crepitans* and *A. gryllis*) collected from ditches and ponds adjacent to heavily treated Mississippi cotton fields were shown to be less susceptible to DDT exposure than those collected from untreated areas. The authors reported that during collection of frogs from these sites, mosquitofish (*Gambusia affinis*) were also frequently collected.

Evidence for DDT resistance in populations of mosquitofish was reported shortly thereafter (Ferguson 1963; Vinson et al., 1963). Other accounts appeared that described populations of mosquitofish that were resistant to other commonly used organochlorine, organophosphate, and pyrethroid insecticides (Boyd and Ferguson 1964; Culley and Ferguson 1969; Fabacher and Chambers, 1973; Ferguson and Bingham 1966; Ferguson and Boyd, 1964; Ferguson et al., 1965; Finley et al., 1970). Reports of cross-resistance (development of resistance to one or more pesticide as a result of exposure to another) were commonly reported (Andreasen, 1985; Culley and Ferguson 1969; Fabacher and Chambers, 1973; Ferguson 1969). Other fish species, including green sunfish (*Lepomis cyanellus*), bluegill sunfish (*Lepomis macrochirus*), golden shiner (*Notemigonus crysoleucas*), and yellow bullhead (*Ictalurus natalis*) inhabiting polluted habitats exhibited chemical resistance, as well (Ferguson et al., 1964, 1965, 1966, 1970). In laboratory studies, guppy (*Poecilia reticulata*) exposed to sublethal concentrations of pentachlorophenol developed resistance similar to that observed in natural populations collected from field sites (Norup, 1972).

Mechanisms of resistance to organochlorine pesticides in fish have not been adequately addressed. Fabacher and Chambers (1973) reported that mosquitofish resistant to the insecticide endrin had higher lipid content than sensitive populations. They speculated that sequestration of toxicants in lipid could reduce toxicity and contribute to resistance. Yarborough and Coons (1975) reported an increase in the number of lipid inclusions in the hepatocytes of resistant mosquitofish, supporting the involvement of lipid reserves in reducing toxicity. In addition to increased lipid content, pyrethroid-resistant fish exhibited higher mixed-function oxidase (CYP-dependent biotransformation) activity (Fabacher and Chambers, 1973). In that study, laboratory-reared offspring of resistant fish exhibited some degree of tolerance. Although CYP activity was not measured in offspring, the authors speculated that elevated CYP might be an inherited trait and a contributor to the apparent genetic adaptation. They also suggested that desensitization of nervous tissue plays a role in resistance, although no supporting data were presented. In another study, Andreasen (1985) reported that the laboratory-reared F_1 progeny of toxaphene-resistant fish maintained a pronounced resistance. The author suggested that resistance was due to natural selection of a genetic trait present in the fish population and that elevated mixed-function oxidase (CYP) may contribute to resistance; however, resistance in generations beyond F_1 were not measured, leaving open the possibility of the involvement of epigenetic mechanisms, maternal inheritance of genetic material, or maternal transfer of the toxicant to progeny. In other mechanistic-oriented studies, Rice and Mills (1987) found no differences in biochemical indicators of hepatotoxicity between kepone-tolerant and -susceptible populations of mummichog (*Fundulus heteroclitus*). Weis and Weis (1989) reviewed much of the early literature on toxicity resistance and concluded that activation of the CYP system during toxicant exposure may contribute to physiological acclimation in some cases. Bonner and Yarborough (1989) provided some evidence that organochlorine-resistant fish may possess an effective central nervous system barrier, leading to reduced target tissue exposure.

Mechanisms associated with the development of pesticide resistance have been investigated more thoroughly in insects (Daborn et al., 2002; Denholm et al., 2002; Guillemand et al., 1998; McKenzie and Batterham, 1994) than in fish. In insects, resistance is often achieved through changes in the structure (e.g., through point mutations) or expression (e.g., through gene amplification) of biotransformation enzymes, especially CYP proteins and GSTs (Oakeshott et al., 2003; Ranson et al., 2002; Taylor and Feyereisen, 1996). Other resistance mechanisms in insects involve alterations in toxicity targets and receptors, including voltage-gated sodium channels and GABA receptors in nerve membranes. (ffrench-Constant et al., 1993; Oakeshott et al., 2003; Ranson et al., 2002; Taylor et al., 1995; Williamson et al., 1993). Abu-Qare et al. (2003) presented several lines of evidence indicating a role for Pgp in the mammalian blood–brain barrier as an effective mechanism preventing accumulation of pesticides in the central nervous system. Although it is likely that tolerance to organochlorine pesticides in fish involves similar mechanisms, there appears to be little active research in this area.

Tolerance to Metals

Evidence for chemical resistance to metals in fish and other aquatic organisms also appeared early in the scientific literature and frequently involved development of resistance in laboratory-exposed populations. Recent investigations have provided insight into processes (acclimation vs. adaptation) and

mechanisms of tolerance (Klerks and Weis, 1987). During evaluation of the toxicity of zinc sulfate to rainbow trout (*Oncorhynchus mykiss*), Lloyd (1960) noticed that preexposure to zinc significantly enhanced median percent survival in subsequent exposures to elevated zinc concentrations. Similarly, Edwards and Brown (1967) found that rainbow trout maintained in a zinc solution were less susceptible to subsequent zinc toxicity as measured by LC_{50} values. By the 1970s, interest in the development of toxicity resistance to metals had become widespread, and numerous efforts were made to better characterize this response. Pretreatment of rainbow trout eggs with zinc (Sinley et al., 1974) or with cadmium (Beattie and Pascoe, 1978) increased the survivability of hatching and larval fish to subsequent metal exposures. Similar results were reported by Spehar et al. (1978) in studies with flagfish (*Jordanella floridae*). In another study, preexposure of larval rainbow trout to sublethal doses of cadmium produced fish with resistance to higher doses of cadmium (Pascoe and Beattie, 1979). Evidence for both physiological acclimation and genetic adaptation began to appear in the literature shortly thereafter; for example, Dixon and Sprague (1981a,b) investigated toxicity resistance in rainbow trout to copper, arsenic, or cyanide. Preexposure to these toxicants resulted in enhanced survivability during subsequent, elevated exposures. Acclimation to copper was lost within 3 weeks of maintenance in clean water, indicating physiological acclimation. Numerous other accounts followed, describing acclimation responses following preexposure to copper, cadmium, mercury, zinc, aluminum, and selenium (Buckley et al., 1982; Duncan and Klaverkamp, 1983; Orr et al., 1986), as well as mixtures of metals (Roch and McCarter, 1984a).

Dixon and Sprague (1981c) provided some of the first mechanistic evidence for the involvement of metal-binding proteins in physiological acclimation to metals. In that study, the authors reported increased synthesis of a low-molecular-weight soluble protein in the liver of copper-tolerant fish. Other investigators subsequently addressed the specific involvement of metallothioneins and concluded that these proteins play important or dominant roles in reducing metal toxicity (Roch and McCarter, 1984a,b). In one study, McCarter and Roch (1983) demonstrated a strong relationship between LC_{50} values for copper in Coho salmon and hepatic metallothionein levels (Figure 13.5).

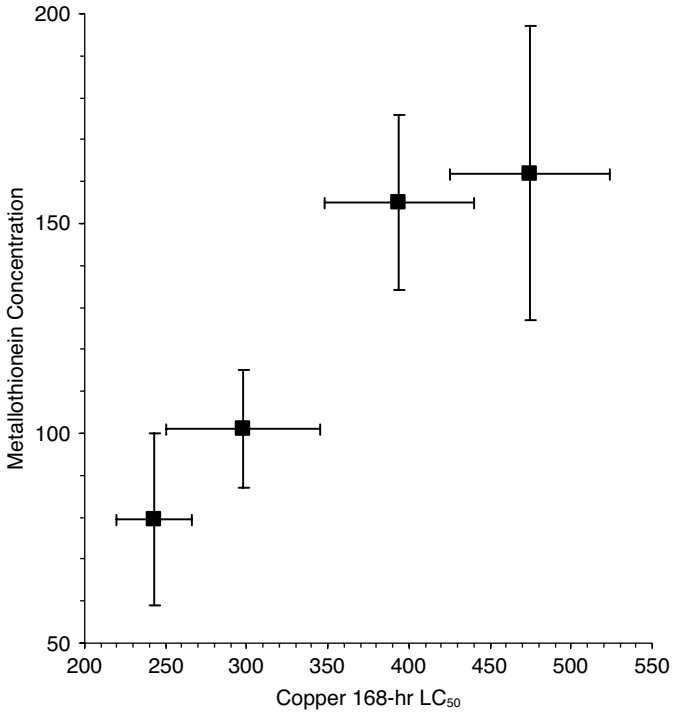

FIGURE 13.5 Plot of 168-hour LC_{50} values vs. metallothionein levels in juvenile Coho salmon exposed to copper. (Adapted from McCarter, J.A. and Roch, M., *Comp. Biochem. Physiol.*, 74C, 133–137, 1983.)

Bensen and Birge (1985) provided some of the most convincing evidence for the role of metallothioneins in physiological acclimation to metals. Fathead minnows (*Pimephales promelas*) collected from a metal-contaminated fly ash pond were much more tolerant of cadmium and copper than hatchery-raised fathead minnows. Maintenance of the metal-resistant individuals in clean water resulted in a significant decline in tolerance within a week. Conversely, tolerance in hatchery-raised individuals increased following exposure to sublethal concentrations of cadmium and declined within a week following their return to clean water. Throughout these experiments, a strong relationship was demonstrated between changes in metal tolerance and expression in the gills of metallothionein or some other metal-binding protein.

In field investigations, Weis and Weis (1977a,b) studied the effects of methylmercury (meHg) and inorganic mercury on embryonic development in mummichog (*Fundulus heteroclitus*), a nonmigratory estuarine species. In early studies, they used eggs from the relatively clean estuarine environment of Montauk and Southhampton on Long Island, New York. Both forms of mercury produced a variety of teratogenic effects, with meHg being more toxic. Later, these authors reported that significant variation in embryonic meHg tolerance occurred between batches of eggs produced by different females (Weis et al., 1982). Furthermore, embryos hatched from the eggs of females from a contaminated estuary (Piles Creek, a tributary of the Arthur Kill in Linden, New Jersey) were much more tolerant of meHg than were embryos from a relatively unpolluted habitat (Weis et al., 1981). These authors presented several pieces of evidence suggesting that meHg resistance in Piles Creek fish represented a genetic adaptation. First, embryos of fish collected from the polluted environment did not exhibit the batch-to-batch variation observed in embryos from the clean environment; instead, embryos from the polluted environment were much more uniformly tolerant to meHg. Second, when female fish from the polluted environment were maintained in clean water, they continued to produce tolerant eggs and embryos. Finally, there was a strong correlation between tolerance and the fin ray count of the female, a characteristic believed to have a large genetic component (Weis et al., 1982).

Recently, Xie and Klerks (2003) evaluated selection for cadmium resistance in multiple generations of laboratory-exposed least killifish (*Heterandria formosa*). Development of resistance occurred within one to two generations and was inherited. After six generations, cadmium-treated fish survived approximately three times longer than control fish when exposed to cadmium. Metallothionein-like protein levels were elevated in cadmium-tolerant lines and likely contributed to observed tolerance (Xie and Klerks, 2004).

Tolerance to Dioxin-Like Compounds

Locations in Newark Bay, New Jersey, and New Bedford Harbor, Massachusetts (Figure 13.6) have been heavily contaminated with DLCs for decades (Long et al., 1995). Much of our understanding of resistance to DLCs comes from studies with mummichog inhabiting the most heavily contaminated locations in these estuaries (reviewed in Elskus, 2001; Hahn, 1998; Hahn et al., 2004; Nacci et al., 2002d; Weis, 2002). In addition, a series of studies by Wirgin and coworkers (Chambers et al., 2003; Wirgin and Waldman, 1998, 2004) suggest that at least one other species of estuarine fish, Atlantic tomcod (*Microgadus tomcod*), has developed resistance to this prevalent and toxic category of pollutants.

Following reports of meHg tolerance in mummichog from Newark Bay, Prince and Cooper (1995a) conducted experiments that revealed differences in the response of Newark Bay progeny and reference-site progeny to TCDD. Fish from the reference site when exposed to TCDD exhibited a higher prevalence of embryonic deformities, lesions, and death (Figure 13.7). These results could not be accounted for by differences in TCDD uptake between groups (Prince and Cooper, 1995b). The authors also demonstrated that adult fish from Newark Bay were unresponsive to EROD induction following TCDD exposure. Downregulation of EROD, a CYP1A-dependent activity, led the investigators to believe that an altered AhR pathway may be involved in resistance to TCDD. In subsequent studies, Elskus et al. (1999) reported no induction of CYP1A or EROD in adult or larvae Newark fish following PCB exposures that caused induction in reference fish. The authors concluded that, consistent with the findings of Prince and Cooper (1995a), Newark Bay fish were resistant to sublethal effects of TCDD and that an altered AhR pathway may be involved in resistance to DLCs.

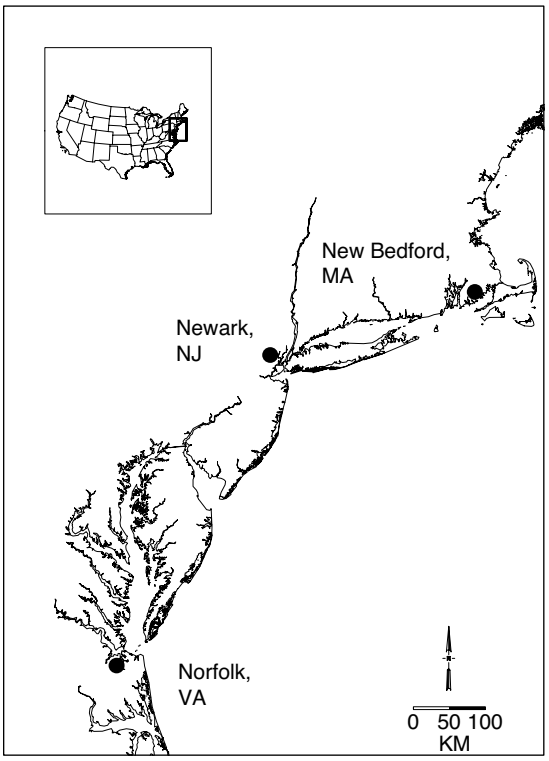

FIGURE 13.6 Map of mummichog (*Fundulus heteroclitus*) study sites along the east coast of the United States. Newark Bay in New Jersey and New Bedford Harbor in Massachusetts are contaminated primarily with DLCs. The Elizabeth River (including the Atlantic Wood site) in Norfolk, Virginia, is contaminated with PAHs and other contaminants associated with creosote.

The toxicity and tolerance of DLCs have also been investigated in mummichog resident to New Bedford Harbor, designated a Superfund site because of extensive and heavy PCB contamination (Lake et al., 1995; Nelson et al., 1996; Pruell et al., 1990; Weaver, 1984). Sediment PCB levels in the most contaminated areas of New Bedford Harbor have been measured as high 190,000 mg PCB per kg sediment or 19% by weight (Weaver, 1984). Mummichog inhabiting sites throughout New Bedford

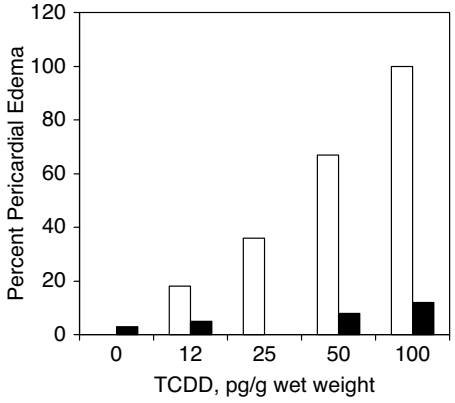

FIGURE 13.7 Prevalence of TCDD dose-dependent pathological lesion formation (pericardial edema) in *Fundulus heteroclitus* embryos from a reference site (white bars) and from Newark (dark bars). (From Prince, R. and Cooper K.R., *Environ. Toxicol. Chem.*, 14, 579–588, 1995. With permission.)

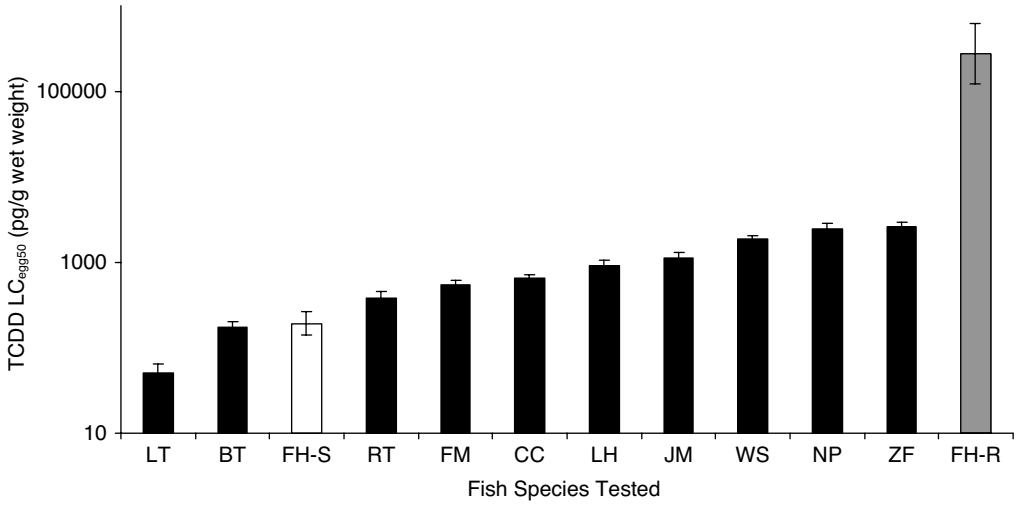

FIGURE 13.8 Variation in response to TCDD (LC_{50} and 95% confidence intervals) in early life stages of fish species (see Figure 13.2) redrawn to include estimated responses of a reference population (white bar) and a tolerant population (New Bedford Harbor, Massachusetts, gray bar) of *Fundulus heteroclitus* (i.e., 193 to 275,964 pg/g dioxin equivalents, respectively, using values from Nacci et al., 2002a, and Van den Berg et al., 1998).

Harbor are heavily exposed to PCBs, including some of the most toxic (dioxin-like) congeners (Black et al., 1998a; Lake et al., 1995). New Bedford Harbor mummichog exhibited levels of specific PCB congeners that were lethal to reference fish when the latter were exposed via injection (Black et al., 1998a) or diet (Gutjahr-Gobell et al., 1999). As a result, Black et al. (1998b) suggested that New Bedford Harbor fish may also exhibit abnormally high mortality in the field; however, a series of studies confirmed that, despite extreme tissue PCB concentrations and modeled predictions of contaminant risks (Munns et al., 1997), New Bedford Harbor mummichogs are reproductively prolific (Nacci et al., 2002d), have high condition indices (Nacci et al., 2001b), and have nearly normal levels of stored vitamin A (Nacci et al., 2001). Vitamin A is considered a sensitive indicator of DLC toxicity in many vertebrate species (Fletcher et al., 2001). Complementary studies suggest that demographic compensation (e.g., increased reproductive effort) and migration (i.e., from less-contaminated populations) do not play important roles in supporting a persistent population in New Bedford Harbor (Nacci et al., 2002b,d, 2007); therefore, tolerance to PCBs was proposed to explain NBH mummichog population persistence.

In subsequent studies with New Bedford Harbor mummichog, Nacci et al. (1999) reported that the acute toxicity of PCB 126 (one of the most toxic DLCs) was approximately 100-fold higher in progeny of reference fish than in progeny of New Bedford Harbor fish. The significance of the difference in sensitivity between populations within a single fish species is revealed when compared to sensitivity variation among fish species for TCDD (Figure 13.2). When LC_{50} values are transformed into toxic equivalency values that reflect sensitivity to TCDD (using values from Nacci et al., 2002a, and Van den Berg et al., 1998), the intraspecies range for this single fish species (mummichog) exceeds the interspecies sensitivity range among tested freshwater fish (Figure 13.8).

Tolerance to DLCs was observed in F_1 and F_2 generations of New Bedford Harbor mummichog and therefore appears to involve genetic adaptation (Nacci et al., 1999, 2002a). Further, concentrations of PCB 126 producing early-life-stage lethality in hybrid embryos (i.e., New Bedford Harbor × reference mummichog) were intermediate in sensitivity between the parental populations, and sensitivities to PCB 126 were similar in hybrids whether of maternal or paternal origins from New Bedford Harbor, suggesting minimal contributions from non-inherited maternal effects (Nacci et al., 1999). Laboratory exposures were transformed into estimates of tissue concentrations and compared to concentrations measured in eggs from New Bedford Harbor mummichog to estimate effects of field exposures (estimated from chemical measurements of sediment cores (Nacci et al., 2002d). These results confirmed that mummichog embryos

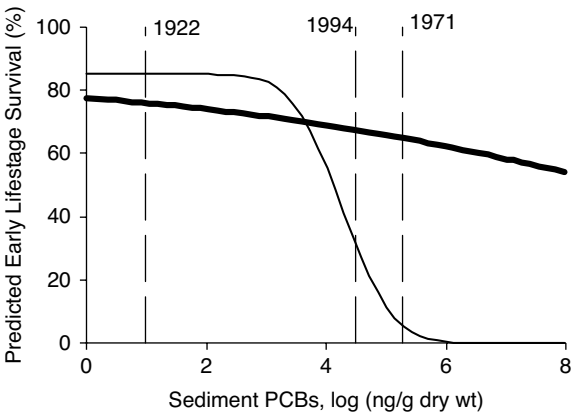

FIGURE 13.9 Models of survival for populations of *Fundulus heteroclitus* from a reference site (light line) and New Bedford Harbor (heavy line) populations based on laboratory studies of toxicity to PCB 126 and bioaccumulation of sediment PCBs (Nacci et al., 2002a). Estimated sediment PCB concentrations based on sediment cores from New Bedford Harbor are shown for dates prior to 1922, at the end of PCB deposition (1971), and in recent times (1994) (Nacci et al., 2002d).

from a reference population, but not native New Bedford Harbor mummichog, would be killed by concentrations of PCB 126 accumulated from New Bedford Harbor sediments (Nacci et al., 2002d). Specifically, PCBs in New Bedford Harbor sediment have been at toxic levels since the 1940s (shortly after the discharge of PCBs into the harbor began), and continue to be toxic, even though discharges of PCBs ceased in the, 1970s (Figure 13.9). Taken together, these studies are consistent with the conclusion that PCB contamination has resulted in a genetically adapted New Bedford Harbor mummichog population.

The results of several studies indicate that DLC resistance in the New Bedford Harbor mummichog involves alterations in responsiveness of the AhR pathway in these fish; for example, reduced responsiveness to DLCs was investigated using a nondestructive *in ovo* EROD assay (Nacci et al., 1998, 2004) (Figure 13.10). Like Newark Bay mummichog, New Bedford Harbor embryos were relatively insensitive to EROD induction by DLCs (Nacci et al., 1999), although there was significant variation in EROD responsiveness among individual embryos within each population (Figure 13.11). Consistent with studies in embryos, adult fish from New Bedford Harbor were also unresponsive to AhR agonists when other endpoints mediated by the AhR pathway were measured. Nacci et al. (2002c), for example, exposed fish to the PAH benzo(*a*)pyrene, an effective AhR agonist and inducer of CYP1A. BaP is also readily biotransformed by CYP1A into mutagenic (DNA adduct forming) products. Exposure of adults to BaP resulted in a lower concentration of BaP–DNA adducts in New Bedford Harbor than reference fish (Figure 13.12). Lower adduct formation in New Bedford Harbor fish could be explained by reductions in CYP1A-mediated activation of BaP to mutagenic forms or enhancement of detoxification (e.g., through GSTs), both consistent with altered molecular mechanisms of the AhR pathway.

Sensitivity to DLCs was also evaluated in cultured hepatocytes from adult New Bedford Harbor fish and reference mummichog (Bello, 1999; Bello at al., 2001). CYP1A was inducible in New Bedford Harbor hepatocytes but required a 14-fold higher concentration of TCDD than that required to induce CYP1A in reference hepatocytes. This indicates that the AhR pathway in New Bedford Harbor parental fish is functional but less sensitive or responsive to AhR agonists than in reference fish. Like parental fish, progeny from New Bedford Harbor were generally refractory to CYP1A induction, suggesting that some mechanisms of tolerance are inherited (Bello, 1999; Bello et al., 2001). Other evidence suggested that short-term or noninheritable mechanisms may contribute to the observed differences between the New Bedford Harbor and reference fish (Bello, 1999); for example, a PAH was a relatively effective inducer of CYP1A, and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was an effective inducer of the AhR in F_1 progeny raised in the laboratory from New Bedford Harbor parents (Bello, 1999). In addition, although freshly caught New Bedford Harbor fish had a lower hepatic content of AhR than reference fish, there were no differences in AhR levels between fish from different sites after a 90-day depuration period in the laboratory.

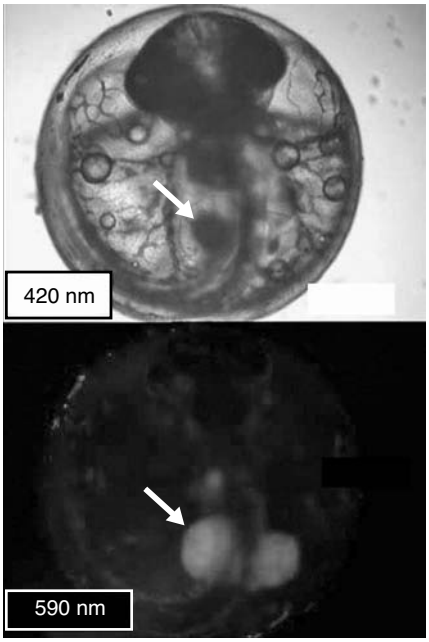

FIGURE 13.10 Photomicrograph showing *Fundulus heteroclitus* embryo from reference population exposed to a dioxin-like compound and provided with the substrate for ethoxyresorufin-*O*-deethylase (EROD) reaction *in ovo*. The bilobed bladder (arrow) fluoresces blue (top panel) at 420 nm excitation and red (bottom panel) at 590 nm excitation.

A series of studies conducted by Hahn and colleagues have addressed variations in structure, regulation, and function of the AhR signal transduction pathway in New Bedford Harbor and reference populations. These studies have focused on the receptor (AhR1 and AhR2), partner (ARNT), and repressor (AhRR) pathway components, as well as the enzymes (e.g., CYP1A) regulated by the pathway. No differences were observed between local reference and New Bedford Harbor mummichog in ARNT expression or AhR2 expression, but an altered pattern of tissue-specific expression of AhR1 was reported in New Bedford Harbor mummichog compared to reference fish (Karchner et al., 1999; Powell et al., 2000). This pattern of expression was not inherited, although resistance in New Bedford Harbor mummichog

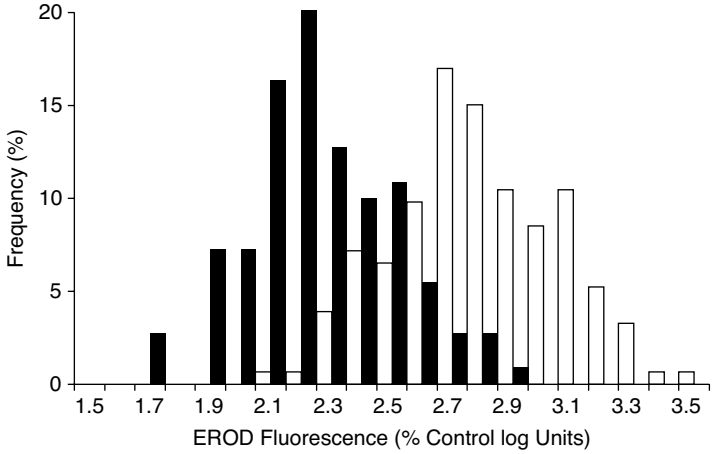

FIGURE 13.11 EROD fluorescence in 3-methylcholanthrene-exposed *Fundulus heteroclitus* embryos; white bars are reference-site embryos; black bars are New Bedford Harbor embryos. (From Nacci, D. et al., *Mar. Biol*., 134, 9–17, 1999. With permission.)

is inherited. Recent studies indicate genetic differences between AhR1 in New Bedford Harbor and reference mummichog. AhR1 in New Bedford Harbor vs. reference mummichog differed genetically but not functionally with respect to binding capacity and affinities for dioxin, suggesting that these differences cannot account for differences in dioxin sensitivity between populations (Hahn et al., 2004).

In addition to AhR1, there appear to be differences in the regulation of AhHR in New Bedford Harbor and reference fish. AhHR is believed to negatively regulate transcriptional activity of the AhR (Karchner et al., 2002; Mimura et al., 1999). DLCs appear to induce AhHR in reference fish but not in New Bedford Harbor fish (Karchner et al., 2002), suggesting that upregulation of AhHR is not involved in resistance. Recently developed molecular tools are being used to explore mechanisms by which mummichog populations have adapted to DLCs; for example, the promoter region of CYP1A was cloned and characterized functionally as containing xenobiotic or dioxin response elements (XREs) and glucocorticoid-response elements (GREs) (Powell et al., 2004). Sensitive, specific polyclonal antibodies were developed for AhR1, AhR2, and AhR repressor protein for mummichog (Merson et al., 2006). Merson and coauthors (2006) described the application of these antibodies to demonstrate the occurrence of AhR pathway proteins in heart, brain, ovary, and other tissues of mummichog. Recent evidence suggests that AhR2 plays an important role in DLC toxicity in zebrafish (Prasch, 2003) and may also do so in mummichog. Taken together, the body of work by Hahn and colleagues has provided a great deal of information on components of the AhR pathway in mummichog and other species, including important clues to the molecular basis for inherited tolerance (genetic adaptation) to DLCs.

Interesting parallels in mechanisms of tolerance have been revealed in studies of another estuarine fish species resident to estuaries contaminated with DLCs. Populations of Atlantic tomcod (*Microgadus tomcod*) inhabiting the Hudson River differ from populations inhabiting less contaminated sites in their responsiveness to toxic pollutants (Wirgin and Waldman, 1998). Like mummichog, tomcod resides in or close to natal estuaries throughout its life cycle and are found in large (10^6) populations (Yuan et al., 2006). Tomcod residing in the Hudson are highly contaminated with DLCs and PAHs and (in some years) exhibit a high prevalence of tumors (Cormier and Racine, 1990). Tomcod collected from the Hudson River and depurated in the laboratory exhibit reduced responsiveness to AhR agonists (Courtenay et al., 1999). In one study, responsiveness was regained in progeny from these fish (Roy et al., 2001); however, recent evidence indicated that Hudson River progeny (F_1 and F_2) were resistant to waterborne PCBs at concentrations that are environmentally relevant to the field site and toxic to reference fish (Chambers et al., 2003). In addition, early developmental gene expression differed in Hudson River tomcod vs. reference populations when similarly exposed to DLCs (Carlson et al., 2003). Specifically, CYP1A and AhRR were not induced in F_1 and F_2 Hudson River tomcod embryos exposed to DLCs (Carlson et al., 2003). Taken together these findings suggest that Atlantic tomcod of the Hudson River estuary have developed resistance to DLCs but not to PAHs, as demonstrated by refraction to CYP1A inducibility (Yuan et al., 2006). This resistance is at least in part heritable (Roy et al., 2001; Yuan et al., 2006) and likely reflective of resistance to the early-life-stage effects of DLCs (Yuan et al., 2001). Furthermore, these tolerant populations occupy a large spatial region, much of which is highly contaminated (200 miles of the Hudson is a Superfund (Yuan et al., 2006).

Other Evidence Involving CYP1A Suppression

From the examples given above, suppression of CYP1A is a common feature of mummichog inhabiting DLC contaminated sites; however, the role of CYP1A and its contribution to toxicity are still unclear and likely system dependent (Schlezinger et al., 2006). Concurrent inhibition of CYP1A, for example, reduces DLC toxicity in mummichog embryos (Wassenburg and Di Giulio, 2004) but not in zebrafish morpholino knock-downs for CYP1A (Carney et al., 2004). Downregulation of CYP1A has been observed in isolated hepatocytes during exposure to oxyradicals (Barker et al., 1994). Oxyradicals can be produced during CYP-dependent biotransformation of contaminants and through redox cycling (see Chapter 6). Reactive oxygen species are produced in marine fish exposed to dioxin-like PCB congeners and are associated with AhR-mediated CYP1A uncoupling and subsequent inhibition (Schlezinger et al., 2006). This uncoupling is characteristic of substrates such as DLCs but unlike some PAH AhR agonists that bind tightly to CYP1A and that are slowly metabolized (Schlezinger et al., 2006). In fish

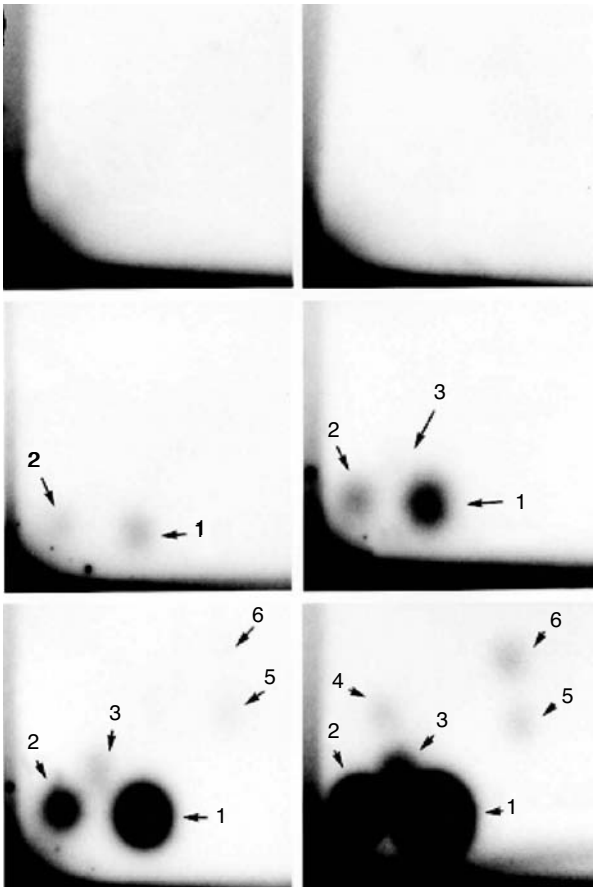

FIGURE 13.12 Autoradiographs of DNA-benzo(*a*)pyrene adducts in livers of *Fundulus heteroclitus* from New Bedford Harbor (left panel) or reference site (right panel). Upper panels are solvent controls; middle panels are 5 mg/kg BAP; lower panels are 50 mg/kg BaP. (From Nacci, D. et al., *Aquat. Toxicol*., 57, 203–215, 2002. With permission.)

embryos, for example, DLC-induced oxidative damage is correlated with CYP1A induction and activity (Cantrell et al., 1998), and embryonic exposure of mummichog to PCB 126 induces CYP1A and ROS production as measured using nondestructive *in ovo* assays for EROD and superoxide detection assays (SoDAs) (Arzuaga et al., 2006).

Several other studies not designed specifically to address toxicity resistance in fish also describe suppression of CYP1A. Some of these have led to alternative hypotheses concerning the mechanisms of CYP1A downregulation and could be relevant to our discussion of resistance mechanisms; for example, perch (*Perca fluviatilis*) collected from Lake Jarnsjon, a Swedish lake heavily contaminated with PCBs, exhibited low levels of CYP1A and EROD compared to those collected from clean sites (Forlin and Celander, 199, 1995). Unlike fish from reference sites, treatment of Lake Jarnsjon perch with PCB 77 or β-naphthoflavone (BNF; a PAH-type inducer) did not result in detectable elevation of CYP1A (Forlin and Celander, 1995). Wirgin et al. (1992) reported that CYP1A in PCB-contaminated tomcod was refractory to induction by PCB 77 but not BNF. These investigators suggested that prior exposure to PCBs but not PAHs affects the subsequent response of CYP1A to DLCs and that differential responsiveness to PAHs and DLCs might also indicate the existence of multiple molecular pathways for induction of CYP1A.

In the laboratory, exposure to high doses of DLCs and PAHs were reported to suppress CYP1A activity (Celander and Forlin, 1995; Celander et al., 1996; Goddard et al., 1987; Hahn et al., 1993; Willett et al., 1998), perhaps through toxicant binding to active CYP1A sites or other mechanisms (Stegeman and

Hahn, 1994). Prolonged (15-week) exposure of rainbow trout to PCBs yielded fish that were refractory to subsequent CYP1A induction by PCBs and the PAH 3-methylcholanthrene (Celander and Forlin, 1995; Celander et al., 1996). Suppression of CYP1A reported in these and other studies (Gooch et al., 1989; Monosson and Stegeman, 1991; White et al., 1997) may itself be related to a form of physiological acclimation to PCBs and other compounds, as suggested by Hahn (1998).

Tolerance to Creosote and Polycyclic Aromatic Hydrocarbons

The Elizabeth River in Virginia is a heavily industrialized subestuary of the lower Chesapeake Bay (Huggett et al., 1992), located in the vicinity of Norfolk and Portsmouth (Figure 13.6). Since the early 1900s, numerous wood treatment facilities along the Elizabeth River used creosote as a biocide to protect telephone poles, pilings, and railroad ties from microbial decay. Although few records exist prior to the 1970s, at least two massive creosote spills occurred in the Southern Branch of the Elizabeth between 1963 and 1967. Elizabeth River sediments exhibit an exponential increase in sedimentary PAHs from the river mouth to approximately 20 km upstream (O'Connor and Huggett, 1988). In addition, Elizabeth River sediments contain other contaminants commonly found in creosote, including nitrogen-, oxygen-, and sulfur- containing heterocyclic compounds, phenols, and pentachlorophenol. PCBs and metals are also present but are considered minor contaminants relative to the aromatic compounds associated with creosote.

While investigating pathologies in Elizabeth River finfish, Vogelbein et al. (1990) discovered a population of mummichog inhabiting a lagoon adjacent to an abandoned wood-treatment facility (Atlantic Wood). Sediment PAH concentrations (2200 mg PAH per kg dry weight sediment) indicated that Atlantic Wood was one of the most heavily contaminated sites known with respect to PAHs and creosote. Adult mummichog at the site exhibited an extremely high prevalence of hepatocellular carcinoma and other lesions (Fournie and Vogelbein, 1994; Vogelbein and Fournie, 1994; Vogelbein et al., 1990, 1999). These lesions were not observed in mummichog collected from a much less contaminated site directly across the river (Scuffletown Creek), suggesting a chemical etiology to these lesions and little migration between sites.

Despite the fact that Atlantic Wood fish develop hepatocellular carcinoma and other chemically induced cancers, they are resistant to acute toxicity associated with exposure to Atlantic Wood sediment (Meyer and Di Giulio, 2002, 2003; Meyer et al., 2002; Ownby et. al., 2002; Van Veld and Westbrook, 1995; Williams, 1994). Laboratory exposures of reference mummichog to Atlantic Wood sediment typically result in visible acute symptoms (i.e., skin and fin erosion, death) within a few days. Under similar conditions, Atlantic Wood fish exhibit no visible symptoms of toxicity. The ability of Atlantic Wood mummichog to tolerate exposure to contaminated sediments is heritable to some extent in laboratory-reared offspring (Meyer and Di Giulio, 2002, Meyer et al., 2003a,b; Ownby et al., 2002). Tolerance declined in the F_2 laboratory progeny relative to that of F_1 (Figure 13.13), suggesting that both physiological acclimation and genetic adaptation contribute to resistance (Meyer and Di Giulio, 2002).

Several studies indicate that Atlantic Wood embryos and larvae are resistant to teratogenic effects of contaminated sediments and individual contaminants. Embryos produced in the lab from reference-site adults and exposed to 100% Atlantic Wood sediment developed severe cardiac malformations (acardia), and exhibited 100% mortality, while embryos from Atlantic Wood parents exhibited only 26% mortality and no cardiac malformations under these conditions (Williams, 1994). Ownby et al. (2002) reported no difference between the first- and second-generation Atlantic Wood mummichog in their ability to resist the teratogenic effects of creosote-contaminated sediment, suggesting a genetic component to resistance. Atlantic Wood embryos also appear to be resistant to teratogenic effects of PCB 126 (Meyer and Di Giulio, 2002). Because PCBs are not present at high concentrations at the Atlantic Wood site, resistance to this DLC suggests that some similarities may exist in mechanisms of resistance to creosote and DLCs.

The mechanistic basis for toxicity resistance in the Atlantic Wood mummichog has been addressed by several investigators. Van Veld and Westbrook (1995) reported low-level expression and insensitivity of CYP1A in Atlantic Wood mummichog to inducing agents, suggesting that alterations in the AhR-mediated pathways contribute to chemical resistance. Lack of CYP1A inducibility persisted in laboratory-

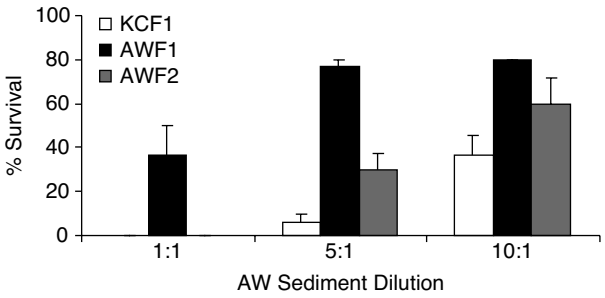

FIGURE 13.13 Survival of Atlantic Wood (creosote-contaminated site) and reference-site (King Creek) mummichog progeny exposed to Atlantic Wood sediment. Progeny were hatched and raised in a clean laboratory environment and exposed for 14 days to Atlantic Wood sediment diluted (1:1, 5:1, 10:1) with reference-site sediment. AWF1, first-generation progeny of Atlantic Wood adults; AWF2, second-generation progeny of Atlantic Wood adults; KCF1, first-generation King Creek progeny. Exposure to 1:1 dilutions resulted in 100% mortality of KCF1 and AWF2. (From Meyer, J.N. and Di Giulio, R.T., *Ecol. Appl.*, 13, 490–503, 2003. With permission.)

reared F_1 embryos and larvae (Meyer et al., 2002); however, inducibility was regained as Atlantic Wood F_1 matured into adults. Further, inducibility was observed in all life stages of later generations raised in the laboratory (Meyer and Di Giulio, 2002; Meyer et al., 2002). This pattern of reversible heritability of the CYP1A phenotype was not explained by maternal effect (Meyer et al., 2002), altered transcription of known CYP1A transcription factors (AHH, AhRR) (Meyer et al., 2003a), or hypermethylation of specific cytosines in the CYP1A promoter region (Timme-Laragy et al., 2005). Differential molecular techniques have also been used to explore pollution tolerance in mummichog from the Atlantic Wood site (Meyer et al., 2005). Following laboratory exposures to sediment from the Atlantic Wood site of fish from tolerant and local sensitive populations, individual variation was high but many sequences were expressed in a sex- and/or population-specific manner, including (potentially) novel genes and those not thought previously to be associated with AhR responsiveness (Meyer et al., 2005).

Exposure to Atlantic Wood sediments caused increases in many of the antioxidant defenses tested in laboratory-raised mummichog from both the Atlantic Wood and a reference-site population (Meyer et al., 2003b). In that study, Atlantic Wood mummichog were more resistant to the toxicity of a model prooxidant than were reference-site fish, and they exhibited elevated levels of several antioxidant defenses in the absence of any chemical exposure. Bacanskas et al. (2004) also noted seasonal and sex-specific responses to prooxidants in pollutant-tolerant and -sensitive mummichog from Virginia residence sites after collection and following laboratory holding. These results suggest that elevated antioxidant defenses might contribute to the resistant phenotype.

Van Veld and coworkers have investigated the relationship between toxicity resistance and cancer in Atlantic Wood mummichog. These fish exhibit altered expression of proteins involved in biotransformation and efflux in a pattern similar to that observed in multidrug-resistant tumors and cancer cell lines; for example, immunoblot analysis indicated reduced expression of CYP1A in hepatocellular carcinoma and in the foci of cellular alteration (Van Veld et al., 1992). Immunohistochemical examination of liver sections revealed similar low levels of CYP1A in hepatocellular carcinoma, exocrine pancreatic tissue, bile ducts, and cholangiocellular proliferative lesions.

Similarities between drug-resistant tumor cells and xenobiotic resistance in Atlantic Wood fish are also suggested by studies with GST. GST activity was elevated approximately fivefold in liver of Atlantic Wood mummichog compared to that of reference-site fish (Armknecht et al., 1996; Van Veld et al., 1991). Monoclonal antibodies against mummichog GST indicated a sixfold elevation of GST in the liver of Atlantic Wood fish relative to that measured in reference fish (Figure 13.14). Sequence information on the elevated GST suggests a relationship to theta-like GST (Van Veld et al., 2005) described in mammals (Mainwaring et al., 1996) and in fish (Gallagher et al., 1999; Leaver et al., 1993).

In addition to alterations in phase I and II enzymes, Pgp transporters were elevated in liver of Atlantic Wood fish relative to reference fish (Cooper et al., 1996, 1999a,b) and were intensely overexpressed in hepatic tumors (Figure 13.15). Immunohistochemical staining of mummichog liver demonstrated this

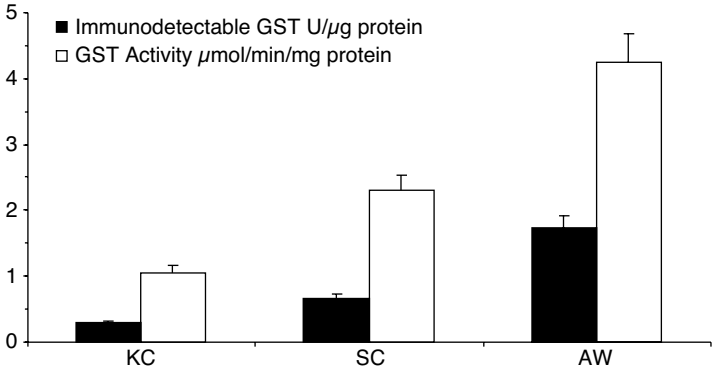

FIGURE 13.14 Immunodetectable glutathione *S*-transferase (GST) and GST activity in King Creek reference-site mummichog (KC), mummichog from moderately contaminated Scuffletown Creek (SC), and mummichog from heavily contaminated Atlantic Wood (AW). (From Armknecht, S.L. et al., *Aquat. Toxicol.*, 41, 1–16, 1998. With permission.)

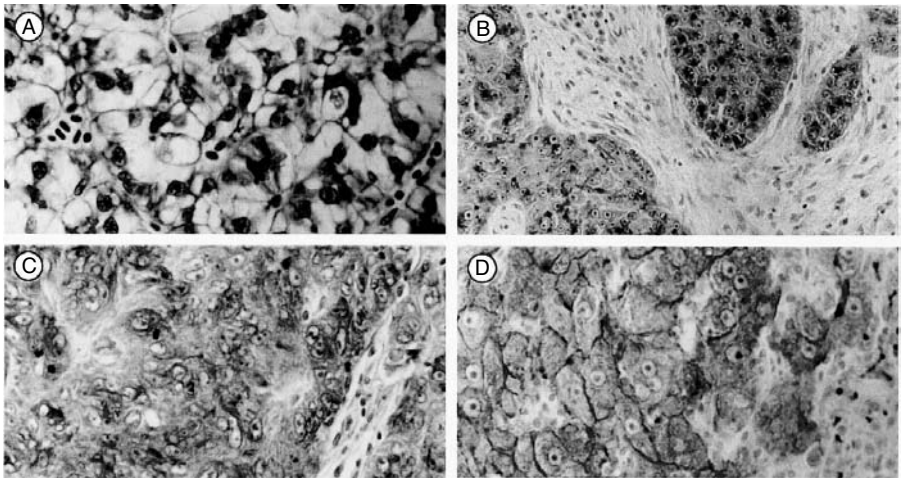

FIGURE 13.15 (See color insert following page 492.) Immunohistochemical staining of mummichog liver demonstrating expression of P-glycoprotein on (A) canalicular surface of normal liver and (B), (C), (D) overexpression in neoplastic cells of tumor-bearing Atlantic Wood mummichog. (Adapted from Cooper, P.S. et al., *Biomarkers*, 4, 48–58, 1999.)

antigen on the canalicular surface of hepatocytes in normal liver (Figure 13.15), suggesting a transport role for this protein. A cDNA fragment was amplified in liver (Cooper et al., 1999b) that was most similar to the mammalian sister gene of P-glycoprotein (Childs et al., 1995). Another cDNA fragment was amplified from mummichog liver and intestine that was a homolog of the mammalian *mdr*, the mammalian multidrug-resistance transporter (Cooper et al., 1999b). These results indicate that a xenobiotic transporter is likely elevated in the livers and liver tumors of a pollution-tolerant vertebrate population, consistent with the proposed role of these proteins in xenobiotic resistance.

Patterns of expression of CYP1A, GST, and Pgp in livers and tumors of Atlantic Wood fish bear a remarkable similarity to patterns observed in mammalian tumors and tumor cell lines. Chemical carcinogenesis as an adaptive response does not explain resistance in Atlantic Wood progeny hatched in clean conditions. Further studies are needed to evaluate the biochemistry of multi-xenobiotic resistance proteins throughout multigenerational life cycles of Atlantic Wood fish in hepatic and extrahepatic tissues. In addition, studies are needed on signal transduction pathways involved in the chemical resistance in tumor-bearing fish. These pathways are still poorly understood in human cancers but appear to involve signaling via activated Ras and tyrosine kinases (Chin et al., 1991; Clark et al., 2003; Ding et al., 2001, Labialle et al., 2002).

Costs Associated with Toxicity Resistance

Ecological and evolutionary theory suggests that adaptation to one form of environmental stress may increase susceptibility to other stressors (Futuyma, 1997). This theory is based on the assumption that adaptation to a new environment involves alterations in a previous, optimal phenotype that has been shaped by the various selection pressures of the ancestral environment (Coustau et al., 2000). Alterations present in the new phenotype should be deleterious to survival in the ancestral environment. Within the context of adaptation to chemical contaminants, traits that are adaptive for specific chemical contaminants may present disadvantages under alternate conditions. These disadvantages are often referred to as *fitness costs* or *fitness trade-offs*. There are several possible mechanisms by which development of toxicity resistance could present disadvantages to adapted individuals (Coustau et al., 2000; Shaw, 1999; Shirley and Sibly, 1999; Taylor and Feyereisen, 1996): (1) Adaptations that involve alterations in cell targets (e.g., ion channels) or receptors could disrupt their normal function in the cell and alter higher level functions in the organism; (2) resistance involving overproduction (induction) of proteins involved in detoxification or sequestration requires energy and may redirect resources away from growth, reproduction, or response to other challenges; (3) genes involved in toxicity resistance might be linked to other cellular processes (i.e., through pleiotrophy) that become unfavorably altered during adaptation to contaminants; and (4) contaminant-induced selection for tolerance may alter population genetic structure and reduce genetic diversity in fish populations. Costs associated with alterations in genetic structure would be dependent on the nature of the alterations themselves.

Despite abundant theory, not a great deal of experimental evidence exists that links toxicity resistance with reduced fitness in vertebrates. Most progress in this area has been made in studies of insecticide resistance in insects where pleiotropic effects of insecticide resistance on putative fitness components have been identified (Raymond et al., 2001). For example, specific alleles involved in pesticide resistance in mosquitoes have been associated with longer development times and shorter wing spans (Bourguet et al., 2004). In addition, single-point mutations conferring target-site mutation resistance to DDT in aphids and houseflies were associated with reduced overwintering survival and decreased response to alarm pheromones (Foster et al., 2003).

Effects of Contaminants on Specific Fitness Components

Experiments designed to study fitness tradeoffs in fish have involved capturing fish from clean and contaminated sites and comparing their responses to various stressors under clean laboratory conditions. Fish that are adapted to contaminated environments often appear less fit in these experiments. Similar observations in insects and plants have been referred to as *between-environment trade-offs*, referring to responses observed after transfer of the study population from the environment to which they have adapted to another environment (Shirley and Sibley, 1999).

Between-environment trade-offs have been observed in studies with creosote-adapted Atlantic Wood mummichog. Poor condition (microbial infection, poor growth, emaciation, and increased mortality) relative to reference fish is routinely observed in Atlantic Wood mummichog maintained in clean estuarine water (P. Van Veld, pers. commun.). Meyer and Di Giulio (2003) reported similar observations, noting poor survival of Atlantic Wood F_1 offspring compared to survival of reference F_1 offspring in artificial seawater. In addition, Atlantic Wood offspring were more susceptible to mortality associated with low oxygen (Figure 13.16) and to toxicity associated with photoactivation of PAHs (Meyer and Di Guilio, 2003). The authors concluded that, although the Atlantic Wood mummichogs are adapted to their contaminated environment, they are maladapted to life in clean environments. The relevance of these and previously mentioned observations to actual field conditions is uncertain. In the field, the Atlantic Wood population appears to thrive and do not appear to be experiencing a great deal of mortality due to low oxygen, phototoxicity, or microbial infection.

Several studies over the past several years have addressed between-environment trade-offs in metal-tolerant fish populations. Laboratory studies indicate that mummichog collected from the methylmercury-contaminated environment of Piles Creek in New Jersey appear to be less capable of capturing natural

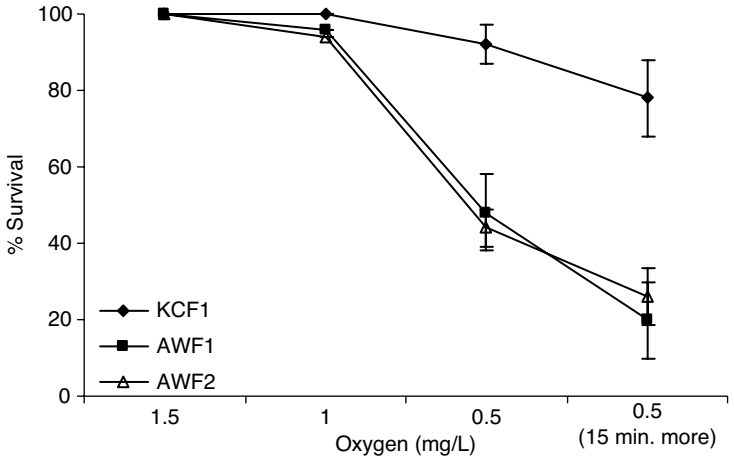

FIGURE 13.16 Larval tolerance of low oxygen conditions in reference-site (King Creek) progeny and Atlantic Wood mummichog progeny. Progeny were hatched and raised in a clean laboratory and exposed to the indicated dissolved oxygen concentrations for 15 minutes. AWF1, first-generation progeny of Atlantic Wood adults; AWF2, second-generation progeny of Atlantic Wood adults; KCF1, first-generation King Creek progeny. AWF1 were significantly less tolerant of low oxygen than were AWF2 and KCF1. (From Meyer, J.N. and Di Giulio, R.T., *Ecol. Appl.*, 13, 490–503, 2003. With permission.)

prey than fish from less-contaminated sites (Smith and Weis, 1997; Weis and Khan, 1991). These studies have established a possible relationship between reduced ability to capture prey with reduced levels of serotonin in brain (Weis et al., 2001) and altered thyroid function (Zhou et al., 1999a,b). Piles Creek mummichog also appear to reach maturity earlier than reference fish but have decreased growth rates and shorter life spans (Toppin et al., 1997). In other studies, Xie and Klerks (2003) reported that laboratory populations of cadmium-resistant least killifish (*Heterandria formosa*) were less tolerant of heat stress than reference populations.

Further studies are needed to determine whether the mechanisms responsible for poor performance of fish in between-environment trade-off experiments are related to toxicity resistance or to other factors stressors, including direct contaminant-mediated effects. Toxic chemicals can exert multiple effects on fish, including adapted individuals, through numerous pathways; for example, abundant literature documents the negative impacts of chemical contaminants on the immune system in fish, including species collected from the Elizabeth River (Rice, 2001) (see Chapter 11). Frederick et al. (2007) reported that Atlantic Wood mummichog exhibit very low titers of antibodies against several common microbial pathogens (e.g., *Vibrio* spp.) compared to the antibody responses observed in reference fish. This observation may help to explain susceptibility of these fish to microbial infection and altered performance in clean laboratory conditions (Meyer and Di Giulio, 2003). In this case, it is unclear whether the altered immune function in Atlantic Wood fish and increased susceptibility to infection is a contaminant-mediated effect or a cost associated with toxicity resistance. Future studies with fish must identify specific genetic loci associated with both resistance and fitness.

Population Genetic Effects of Contaminant Exposure

Evidence exists that exposure to environmental contaminants can alter genetic diversity and population structure in marine organisms (Nadig et al., 1998; Nevo et al., 1984; Theodorakis et al., 2003). Because genetic adaptation involves selection for tolerant genotypes, adaptation could result in the loss of sensitive genotypes and reduced genetic diversity. Evolutionary theory suggests that populations exhibiting decreased genetic diversity may be less suited to function in variable environments because of altered physiological efficiency, viability, fecundity, mating success, or resistance to disease (Mitton, 1997).

Several protein and nucleic acid methods have been used to assess genetic diversity and population structure in diverse organisms. Allozymes are protein variants encoded at the same genetic locus but

differing in amino acid sequence. Allozymes used in population genetic studies are generally those involved in biochemical respiration. Variation observed in stressed populations is therefore not presumed to be mechanistically linked to survival, but may co-occur with genes linked to survival. Nucleic acid techniques involve amplification and detection of random, repeated, or known gene fragments and comparing their occurrence in different fish populations (Bagley et al., 2001; Sunnucks, 2000).

Numerous field studies support the hypothesis that environmental contaminants can alter population structure and genetic diversity in fish. Reduced genetic diversity has been demonstrated in fish populations associated with a uranium processing facility in Ohio, a coal-ash settling basin in South Carolina, and an acid-impacted drainage in New York (Guttman, 1994). Murdoch and Hebert (1994) reported reduced mitochondrial DNA variation in brown bullhead from contaminated sites relative to those from less contaminated sites in the Great Lakes. Gillespie and Guttman (1989) found significant differences in allele frequencies in populations of the central stoneroller (*Campostoma anomalum*) from sites above and below a contaminated region of a stream.

Kopp et al. (1992) analyzed genotypic frequencies in central mudminnow (*Umbra limi*) populations from acid-stressed and nonacid-stressed sites and found that for several loci the stressed populations were characterized by a much greater frequency of one particular allele. The results suggested that environmental conditions were acting as selective forces; however, these data must be interpreted cautiously, because population differentiation can result from processes other than pollutants acting as selection agents (Diamond et al., 1991; Gillespie and Guttman, 1989; Kopp et al., 1992; Mulvey and Diamond, 1991). Studies of 20 polymorphic loci in mummichog, for example, revealed significant directional changes in gene frequencies with latitude (Cashon et al., 1981: Powers and Place, 1978; Powers et al., 1986; Ropson et al., 1990). Thus, although different allozyme patterns were found in populations of mummichog from polluted and nonpolluted sites in a study by Heber (1981), these differences were associated with the north–south pattern in genotypes of this species and apparently were not pollution related.

The hypothesis that changes in the genetic structure of a population can be attributed to environmental contamination is supported by results of several laboratory exposure studies. Gillespie and Guttman (1989) found that stonerollers with genotypes that appeared to be sensitive to contaminants in the field also appeared to be more sensitive to copper toxicity in laboratory tests. Chagnon and Guttman (1989) exposed laboratory stocks of mosquitofish to copper and cadmium and found survival to vary significantly with different allozyme genotype and specific alleles. Diamond et al. (1989) and Newman et al. (1989) examined genotypic frequencies at eight enzyme loci during acute exposure of mosquitofish to arsenate or inorganic mercury and found that time to death was related significantly to genotypes at two of the eight loci for arsenate and three of the eight loci for inorganic mercury. Other investigators reported differential survivorship among mosquitofish genotypes following exposure to mercury (Diamond et al., 1989), arsenate (Newman et al., 1989), and cadmium (Chapman and Guttman, 1989). Differences in time to death were also reported in different genotypes of mosquitofish and sand shiner (*Notropis ludibundus*) exposed to the pesticides lindane and parathion (Sullivan and Lydy, 1999).

Mummichog in the Elizabeth River in Virginia and New Bedford Harbor in Massachusetts have provided opportunities to evaluate the effects of contaminant exposure on genetic diversity and population structure in populations of DLC- and creosote-resistant mummichog. Allozyme analyses were conducted in mummichog collected from sites in the Elizabeth River and York River that vary by several orders of magnitude in concentrations of PAHs (Mulvey et al., 2002, 2003). Fish from the most heavily contaminated site (Atlantic Wood) were genetically distinct from fish collected from other Elizabeth River sites. The data suggested that a locally stable population existed at the Atlantic Wood site; however, no evidence for decreased genetic diversity in this population was observed. Further, no relationship was observed between sediment PAH concentrations and genetic diversity in fish collected from any of the study sites.

Similar results were reported in studies with New Bedford Harbor mummichog (McMillan et al., 2006; Roark, 2003). Differences in the genetic structure of the populations under investigation were related to the geographic distances separating them. No relationships were observed among genetic structure, sediment contamination at the collection sites, and PCB tolerance in the populations inhabiting those sites. Genetic diversity did not differ significantly between sites, and the New Bedford Harbor fish did not exhibit a reduction in genetic diversity relative to those collected from relatively clean sites. In

fact, large historical effective population sizes and no evidence of recent population bottlenecks were found for 15 mummichog populations resident to sites along the Atlantic coast of the United States, including New Bedford Harbor (Adams et al., 2006).

A review of the published literature suggests that the effect of environmental pollutants on genetic diversity is mixed (van Straalen and Timmermans, 2002). Some researchers suggest more rigorous experimental designs are required to establish relationships between genetic alterations and environmental contamination (Belfiore and Anderson, 1998; Theodorakis, 2003). Hebert and Luiker (1996) concluded that no studies in feral fish have demonstrated conclusively that contaminant exposures have produced selection pressure "strong enough to purge populations of their diversity." Clearly additional research is needed before we can use a population genetics approach to properly evaluate costs associated contaminant exposure or toxicity.

Studies Involving Variation in Specific Genes

Population genetic approaches typically involve identification of variation in genes unrelated to adaptation or other responses to contaminant exposure. Genomic variation at specific loci would be useful to identify variation in genes that are involved in chemical adaptation. Candidate genes for adaptation in New Bedford Harbor mummichog include those believed to be associated with toxicological responses to DLCs (e.g., AhR signal transduction pathway). In New Bedford Harbor mummichog, AhR1 is highly polymorphic in loci of the ligand-binding regions (Hahn et al., 2004). The adaptive implications of these genetic changes were investigated using *in vitro* transcription and translation systems; however, simple and clear differences between tolerant and sensitive mummichog populations were not revealed (Hahn et al., 2004). Regions of AhR1 vary between New Bedford Harbor and reference fish, but these variants do not differ in binding capacity, affinity for TCDD, or ability to support TCDD-dependent transactivation (Hahn et al., 2004). Ongoing studies are investigating genetic variation and their functional implications in other regions of the AhR1 (i.e., regulatory region) (S. Cohen, pers. commun.) and other proteins of the AhR signaling pathway (i.e., AhR2, AhRR) (M. Hahn, pers. commun.).

In addition to those genes associated with toxicological responses to pollutants, other genetic loci may be subject to selective pressures and adaptive responses in animals surviving highly degraded environments; for example, the major histocompatibility complex (MHC) is a component of the vertebrate adaptive immune system. Genetic variation in MHC loci appears to vary in association with specific diseases (Coltman et al., 1999). Major differences have been observed between amino acid substitution patterns at the MHC antigen-binding locus of New Bedford Harbor and reference-site mummichog (Figure 13.17) (Cohen, 2002; Cohen and Nacci, 2002). New Bedford Harbor mummichog and reference-site mummichog also exhibit increased prevalence and rare types of parasite infestation (Cohen et al., 2006). In addition, New Bedford Harbor mummichog and their progeny survive laboratory challenges to *Vibrio harveyii* better than reference fish (D. Nacci, unpublished data). Whether variation in MHC represents an adaptation to parasitic or microbial challenge in New Bedford Harbor fish has not been determined. Additional questions concern the role that chemical stress and habitat degradation may play in host–parasite exposures, host–parasite cycles, and variation in MHC; for example, reduced parasite loads in mummichog from Piles Creek in New Jersey, relative to a reference site, were interpreted as a sign of a disturbed ecosystem (Hudson et al., 2006; Schmalz et al., 2002).

Evolutionary and Ecological Impacts of Resistance

Aquatic environments modified by intense human industrial and agricultural activities are typically characterized by physical disruption (e.g., channelization), loss of habitat, excessive nutrient and sediment enrichment, and elevated levels of diverse contaminants. Because any these factors can contribute to alterations in community structure and function, it is difficult to evaluate the relative contribution of any single stressor to changes observed at degraded sites; however, demonstration and characterization of toxicity resistance may allow identification of relationships between: (1) specific biochemical and molecular responses (biomarkers) of toxicity and resistance, (2) survival of tolerant species, and (3) loss of sensitive species. Thus, toxicity resistance to specific contaminants in specific species inhabiting these

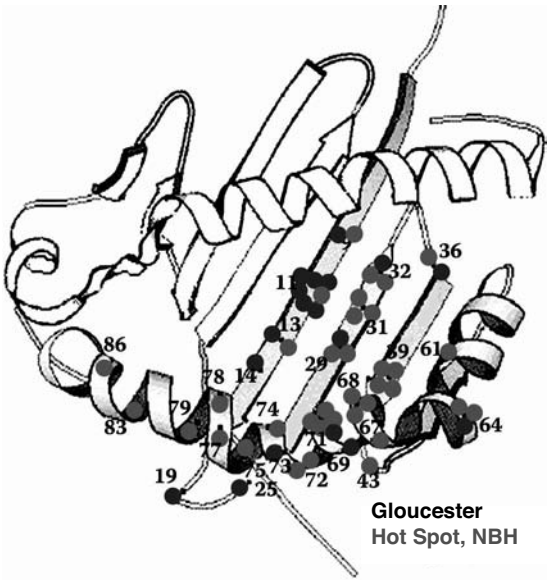

FIGURE 13.17 MHC class IIB structural diagram with inferred locations of population-specific amino acid changes superimposed for a reference population (Gloucester, black) and New Bedford Harbor (gray). Each colored dot represents a single population-specific amino acid change. (Adapted from Cohen, S., *Mol. Biol. Evol.*, 19, 1870–1880, 2002.)

sites may be one of the most informative observations regarding the contribution of contaminant exposure to altered community structure.

Chesser and Sugg (1996) developed an ecosystem dynamics model to evaluate the impacts of selective agents on populations and community dynamics. These authors considered the roles that species redundancy, adaptation, and immigration play in the ability of an ecosystem to cope with toxic insults. Toxic exposures that threaten sensitive genotypes and species can change both the structure (number of species and population sizes) and the function (energy flow) of an ecosystem. The loss of sensitive species and genotypes results in simplified ecosystems that are less predictable and demonstrate lowered resiliency and lowered ability to rebound from subsequent perturbations.

Other disturbances at the ecosystem scale and beyond may be associated with the occurrence of chemically adapted fish. Fish inhabiting contaminated sites can become vehicles for the transfer of toxic chemicals through the food web. This may be particularly important with persistent, lipophilic contaminants (e.g., organochlorine pesticides and DLCs) because of their tendency to accumulate in fish. Although trophic transfer is not restricted to adapted populations of fish, adapted individuals may be capable of accumulating and transferring higher levels of toxicants than nonadapted individuals (Figure 13.18). Transfer of chemicals from adapted individuals to nonadapted individuals can be lethal. In one study, for example, insecticide-resistant sunfish tolerated a diet of insecticide-resistant mosquitofish containing concentrations of endrin that killed control sunfish fed the same diet (Ferguson et al., 1966). In another study, a single resistant mosquitofish eliminated enough endrin into 10 liters of water to kill all control mosquitofish maintained in that water (Culley and Fergusen, 1969). Trophic transfer can result in the bioaccumulation of contaminants in top predators, fish-eating birds, and other wildlife that use the ecosystem as a feeding ground.

Recent concern has arisen over the acceleration in the evolution of drug-resistant disease organisms and pesticide-resistant insects (Palumbi, 2001). Documented cases of toxicity resistance in fish appear to primarily involve populations of nonmigratory fish species limited to specific highly contaminated sites; however, considerable movement and breeding between local populations of fish is expected. Increases in the frequencies of resistance genes could therefore increase the frequency of deleterious alleles in the gene pool of nonadapted populations. In addition, it is likely that fish inhabiting moderately contaminated sites or migratory species that pass through contaminated areas seasonally may experience contaminant-

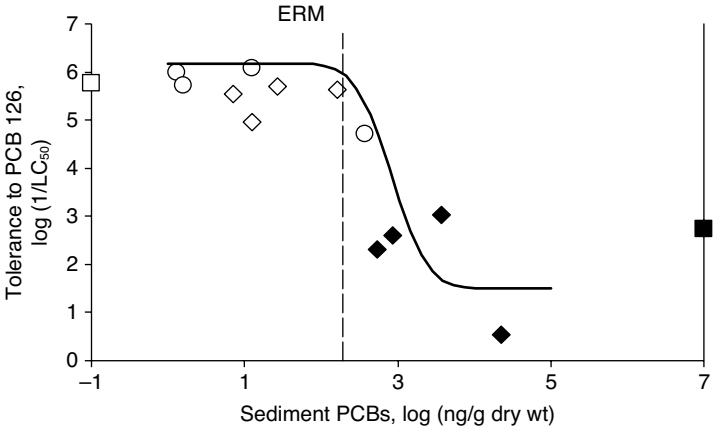

FIGURE 13.18 Measured tolerance ($1/LC_{50}$) for PCB 126 for 14 populations of *Fundulus heteroclitus* vs. sediment polychlorinated biphenyl (PCB) concentrations at each collection site. Dark symbols indicate sites in highly contaminated regions of New Bedford Harbor, Massachusetts (triangles), and Newark, New Jersey (squares). Predictive model for tolerance in *Fundulus heteroclitus*, solid line; sediment PCB guideline value for adverse ecological effects (effects range median, 190 ng/g dry weight) (Long et al., 1995). (From Nacci, D. et al., *Environ. Toxicol. Chem.*, 21, 1525–1532, 2002. With permission.)

mediated selection pressures that go unnoticed. In insects, selection for tolerant individuals appears to be associated with numerous costs related to survival and life-history strategies. The long-term consequences of continuous chemical exposure in populations of fish are unknown but could include similar costs.

Summary and Conclusions

Populations of tolerant fish chronically exposed to environmental contaminants have provided opportunities to characterize resistance and to address underlying mechanisms. For those species that cannot avoid exposure to complex mixtures of toxic chemicals, survival is likely dependent on a battery of defense mechanisms. Physiological acclimation and genetic adaptation have been used to describe short-term reversible responses and genetic-based tolerance, respectively. Other processes including chemical carcinogenesis and epigenetic mechanisms may contribute to tolerance as well.

Tolerance can have profound effects on the ability of fish to survive exposures that are lethal to nonadapted individuals. Within-species variation related to history of exposure can be as large as variation between fish species. Attributes of the exposed fish species (e.g., demography, life history, reproductive strategy) and attributes of the contaminant involved (e.g., toxicity mechanism, toxic potency, exposure frequency, duration, magnitude) are important factors that should be considered when considering tolerance in specific populations.

Much of our understanding of resistance mechanisms in diverse organisms comes from the fields of medicine (drug and antibiotic resistance) and agriculture (pesticide resistance in insects). The highly conserved nature of the pathways involved will allow us to continue learn from these systems. General mechanisms by which tolerance is produced often reflect alterations in pathways by which toxicity is exerted; for example, modification of a cellular receptor can result in reduced toxicant–receptor interaction, resulting in reduced toxicity. Increased tolerance might also be the result of altered regulation of proteins involved in toxicant sequestration (binding), toxicant biotransformation (degradation), or efflux (elimination). Our knowledge on mechanisms of resistance in fish to specific chemicals can be briefly summarized as follows:

- *Organochlorine pesticides*. Although some alterations (altered biotransformation, altered sequestration) in biochemical systems were proposed in the early literature, the underlying mechanisms of resistance to organoclorine pesticides in fish are poorly understood. This area

of research needs to be revisited in light of recent advances in technology and advances in our understanding of specific resistance mechanisms in insects and mammals (e.g., altered biotransformation, altered toxicity targets).

- *Metals*. Investigations of metal tolerance in fish populations have provided insight into processes (i.e., acclimation vs. adaptation) and mechanisms of tolerance to metals. Most of these studies indicate a strong relationship between tolerance to metals and upregulation of metal-binding proteins, especially metallothioneins.
- *Dioxin-like compounds*. A great deal of recent effort has focused on populations of mummichog inhabiting sites (e.g., New Bedford Harbor) contaminated with dioxin-like compounds (DLCs). A large body of evidence indicates that one or more components of the aryl hydrocarbon receptor (AhR) pathway are altered in these fish. Although the specific alterations in this pathway have not been identified, continued active research in this area of research will likely lead to success.
- C*reosote/PAHs*. An altered AhR may also play a role in resistance of Atlantic Wood mummichog to compounds associated with creosote. In addition, a pattern of protein and enzyme expression resembling multidrug resistance in cancer suggests that a similar multi-xenobiotic-resistant phenotype may contribute to survival in this population. These fish provide a model for studies of multidrug (multi-xenobiotic) resistance that may have relevance to other exposed vertebrate species.

Adaptation to chemical stress may come at a cost. Several recent reports suggest that adapted fish maintained in clean laboratory environments have a reduced ability to respond to environmental stressors (e.g., low oxygen, microbial exposure) or to perform (e.g., capture prey) when compared with responses in observed normal individuals. Further studies are needed to establish specific relationships (e.g., pleiotropic relationships) between resistance and performance in actual field situations. Expected associations between reduced genetic diversity and genetic adaptation may require reconsideration in light of observations made in adapted mummichog populations that maintained a high level of genetic diversity.

Current research on toxicity resistance in fish has focused on a number of opportunistic fish species that reside for most or all of their lives in highly contaminated sites. Future studies should include investigations of the abilities and limitations of more sensitive species that may be capable of adapting to moderately contaminated environments. These studies should focus on sensitive toxicity endpoints such as reproduction, development, and behavior. Additional studies are needed on emerging contaminants (e.g., personal-care products, pharmaceuticals, wastewater organic contaminants) that likely exert subtle effects during long-term, low-level exposures.

Tolerance has important consequences relative to population persistence and ecosystem function. The mummichog has been the subject of extensive investigations on adaptation to environmental variables (e.g., temperature) along large geographic and geological time scales (Place and Powers, 1979; Powers and Place, 1978; Powers et al., 1986). Studies on the effects on mummichog of chronic exposure to persistent chemical contaminants will ultimately lead to a better understanding of the impacts of these stressors on evolutionary processes. Genomic tools under development for this species will advance progress in this area (Burnett et al., 2007). Additional research is also needed on physical and biological environmental factors and on species attributes, such as genetic variation and life-stage-specific dispersal characteristics, that may contribute to the development, maintenance, and costs of adaptation to toxic pollutants.

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Unit III

Methodologies and Applications

14

Exposure Assessment and Modeling in the Aquatic Environment

Donald Mackay and Lynne Milford

CONTENTS

Introduction

In aquatic and marine systems, the ultimate toxic effects exerted by chemical substances or toxicants on fish and other organisms are determined by a combination of chemical concentration, exposure time, and conditions. These stresses are, in turn, determined by a sequence of events starting with the loading or discharge of the toxicant to the system which can be expressed in kg or kg/year and may originate from point sources such as industrial or municipal effluents, non-point sources such as atmospheric deposition or terrestrial runoff, and advective inflow from rivers or tributaries. Also, in-place toxicants originating from previous discharges may be found in sediments. The total quantity of introduced toxicant is then subject to fate processes, including transformations and partitioning between all the phases present, resulting in concentrations being established that dictate doses and thus cause effects. In this chapter, we focus on techniques for estimating the masses, concentrations, and fate of chemicals in aquatic systems. In principle, if an undesirable effect occurs as a result of a high concentration, the obvious approach is to reduce inputs of the substance with the expectation that concentrations will fall. The problem is often to determine the magnitude of the desired reduction and how long it will take for improvements to be evident.

In Chapter 2 of this book, Ankley et al. emphasized not only the need for information on the total quantity of toxicant present in an aquatic system but also the importance of quantifying where it will be found (i.e., the extent to which it partitions between the water column, suspended solids, sediment, and possibly to dissolved or colloidal organic and inorganic material). The toxicant may also speciate chemically into ionic and non-ionic forms and generate other chemical complexes. The net result is that, whereas it is obviously essential to ascertain the total concentration of toxicant in the environment, it is also necessary to determine how the toxicant is distributed among a variety of states with differing bioavailabilities (van Brummelen et. al., 1998). This information helps to determine the rates of uptake

by fish and other organisms by diverse routes such as respiration, food ingestion, and dermal absorption. It is important to determine and quantify the dominant routes of uptake that control the *dose* received by the fish, which in turn ultimately controls the *effect* of the toxicant. For most organic toxicants, the chemical is absorbed from food or respired water into the bloodstream and becomes distributed among organs and tissues, especially those of high lipid content. Metals, on the other hand, may specifically partition to respiring surfaces, resulting in interference with critical transport processes.

When a toxicant is suspected of having adverse effects on a fish population, a standard response is to sample and analyze the water or sediment and infer the likelihood of effects by comparing measured concentrations with those known to cause adverse effects in laboratory exposures or bioassays. Unfortunately, such analyses are often fraught with difficulties. First, a satisfactory analytical method for measuring the concentration may not be available; for example, only recently have methods become available to analyze the alkyl phenolic substances described by Tyler in Chapter 25. Appreciable spatial and temporal variability in concentrations may occur due to the nature of the environment. Also, such analyses may not adequately discriminate between a truly dissolved organic substance and that which is associated with dissolved matter such as fulvic acids. These two different forms found in the environment are likely to differ in bioavailability. Finally, chemical analyses of these type can be prohibitively expensive for the amount of useful information gained.

When the presence of a toxicant is established and approximate levels are known, it may be useful to supplement measurements by *mass balance models*, which provide a basis for calculating the concentrations, partitioning, and process rates in the aquatic system. These deductions should be validated by comparing predictions with actual measurements, but in some cases the only method of estimating concentrations is by calculation. A major benefit of compiling a comprehensive, validated mass balance model is that it can provide a complete picture of the fate of the toxicant, including estimates of certain quantities such as evaporation rates that cannot be measured directly. Such a model allows predictions to be made of the effect of reducing loadings (namely, by how much) and how quickly concentrations will fall. The model may help to explain seasonal variations of concentration, bioavailability, and exposure. It is becoming increasingly evident that environmental management or regulation to restore viable fish populations with minimal contamination is greatly assisted by having available a full, quantitative description of the sources and fate of the toxicant and, accordingly, the actual exposure experienced by aquatic biota.

A large number of models is available, many of which can be downloaded from the Internet and are supported by agencies such as the U.S. Environmental Protection Agency (EPA). It is impossible to describe these models in detail, so our focus here is to present and illustrate the basic principles that apply in a simple single-box model and to outline briefly how this approach can be extended to treat more complex systems. We emphasize that models are necessarily simplifications of a complex reality; thus, they are no substitutes for direct measurement. The equations inherent in the model represent the modeler's best attempts to express the phenomena quantitatively, but these expressions may be incomplete or flawed. All models rely on estimates of input parameters such as temperature, flow rates, rate constants for degrading reactions, and partition coefficients expressing equilibrium concentration ratios between phases. All are subject to variation, error, and uncertainty. The result is that the model output is rarely more accurate than a factor of 2 or 3. The modeler has an obligation to convey this uncertainty to the user to avoid over-interpretation of the results or over-reliance on the findings. Models are entirely complementary to monitoring efforts, not a substitute for them. It is, however, very satisfying when agreement between the two occurs, as this suggests that the system is both reliably monitored and quantitatively understood.

A Historical Note

It is appropriate to provide a brief historical perspective on mass balance modeling of aquatic systems and to acknowledge the pioneering efforts of early workers in this field. These models date back to the 1920s, when it was recognized that the discharge of degradable organic wastes into rivers was depleting dissolved oxygen levels and causing fish mortality. The studies by Streeter and Phelps (1925) were the

first to characterize the process quantitatively in the form of the *oxygen sag* equation, which is essentially a riverine oxygen mass balance. Later, these principles were applied to lakes and estuaries and to other substances, notably hydrophobic organics, pesticides, metals, radionuclides, and nutrients. Much of the pioneering work in North America was done at Manhattan College in New York by O'Connor and his colleagues, notably Mueller, Thomann, Di Toro, and Connolly. Thomann (1998) reviewed the evolution of these models as they have grown in scope, complexity, and utility. The models now routinely treat water, sediment, the atmosphere, and aquatic food webs by segmenting the system into numerous connected boxes and allowing for time-dependent changes in hydrodynamics and temperature. Episodic events such as spills can be simulated. Thomann (1998) suggested that we are now entering a "golden age" of aquatic modeling with the potential to contribute to the rational and effective management of aquatic and marine systems. Currently available models are designed to treat organic substances, metals, nutrients, and radionuclides. They vary greatly in complexity. They can be applied to lakes, rivers, wetlands, estuaries, groundwater, and oceanic systems. Some include estimates of bioaccumulation and toxicity. They all have the common objective of seeking to describe or simulate the fate of the chemical in an aquatic system.

Fundamental Concepts

The simplest aquatic system unit is a small lake that consists of a well-mixed body of water, underlain by a layer of well-mixed sediment with an overlying atmosphere. Use of the concept of well-mixed compartments enables the amount of toxicant to be characterized by a single concentration rather than a distribution of values. This is obviously a simplification, but it greatly facilitates mathematical simulation. The water column is usually treated as consisting of water, suspended solids, and, in some cases, dissolved organic matter. Discrimination between *dissolved* and *suspended* is operationally defined by the pore size of a filter (usually a fraction of a micrometer); thus, smaller particles such as fulvic and humic acid agglomerations may be treated as being dissolved when in reality they are separate phases. The bottom sediment is usually treated as a mixture of water, mineral solids, and organic matter. Vertebrate and invertebrate biota may be present in both water and sediment. Finally, the atmosphere above the lake is treated as consisting of air and aerosol particles with chemical transport facilitated by rainfall.

Toxicants can move through this system via two general transport processes. Any toxicant associated with particles in air, water, or sediment will move with these particles as they deposit, resuspend, or flow with the water. Toxicant will also flow with the water in dissolved form. These are essentially *advection* processes. In addition, the toxicant is in a continuous state of *diffusion* through the system, with a tendency to approach thermodynamic equilibrium (i.e., equal chemical potential or fugacity). Concentrations in water and air, for example, will tend to approach equilibrium as dictated by Henry's law. At all times, the substance is subject to *transformation* or *degradation* processes such as microbial conversion, photolysis, hydrolysis, or oxidation in all media.

In most, but not all, cases the mass balance model can ignore biota (except for biodegradation) because the mass of substance associated with biota is usually insignificant. Exceptions would include the transport of chemicals associated with migrating salmon or bird guano. The concentration in biota can be large multiples of that in water, but because the volume of biota is relatively small (e.g., 1 to 10 parts in 1 million) the mass in biota is also small. This simplifies the model by allowing the dominant abiotic fate processes to be deduced. Concentrations in biotic phases such as fish can be estimated later in a separate calculation.

Figure 14.1 illustrates the processes to which a substance may be subjected in a simple unit lake. Table 14.1 lists these processes and suggests the structure of the equations used to describe the equilibria or kinetics. The modeler's objective is to write equations describing all these processes in terms of the local concentration of the substance, combine them, and then solve the overall mass balance. This can be viewed as an accounting problem in which the aim is to balance the numerous inputs and outputs.

Different modelers write the various expressions describing the rates of these processes in different ways; however, they share the common goal of quantifying all process rates accurately. These expressions

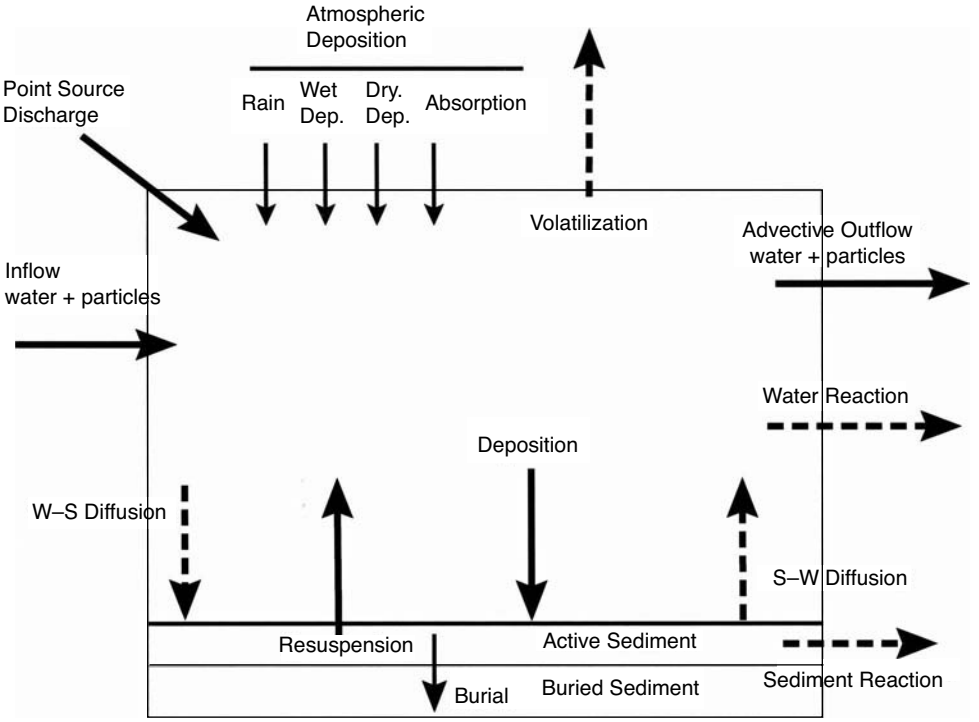

FIGURE 14.1 Input and output processes that are quantified by the Quantitative Water Air Sediment Interaction (QWASI) model.

take a variety of forms; for example, a rate of outflow in units of g/hr can be expressed as the product of a water flow rate (m^3/hr) and a concentration (g/m^3). A reaction rate can be expressed as the product of a volume (m^3), concentration (g/m^3), and rate constant (hr^{-1}), the latter being 0.693/half-life (hr). A volatilization rate can be the product of an area (m^2), concentration (g/m^3), and mass transfer coefficient or velocity (m/hr). The resulting mass balance equations become quite lengthy, and extreme care must be taken to ensure that units are consistent. The model is likely to be applied to a variety of toxicants that differ in properties. The expressions must allow the user to input these various properties while ensuring that the equations remain valid; that is, the model should be robust enough to accept these inputs and treat them correctly.

An alternative and ultimately equivalent approach is to use the concept of *fugacity* as a surrogate for concentration, as described by Mackay (2001). The concentration (C) (mol/m^3) is expressed as a product of fugacity f (Pa) and a Z value (mol/Pa m^3). Fugacity can be regarded as partial pressure, or escaping tendency, of a substance. The Z value is specific to the substance and the medium in which it resides and can be viewed as a kind of solubility. Z values are deduced from equilibrium partition coefficients. A partition coefficient K_{12} between two phases 1 and 2, such as air and water, can be shown to be Z_1/Z_2. Usually, Z is first defined for air, then the values for other phases such as water, sediment, and fish are deduced. All process rates (mol/hr) are expressed as the product of a fugacity (f) (Pa) and a D value (mol/Pa hr). Fugacity-based and concentration-based equations are essentially identical, but the fugacity equations are more compact. D values are best thought of as rate constants for the process expressed in terms of fugacity. Applying the list of D values in Table 14.1 to the diagram in Figure 14.1 and defining fugacities for the chemical in air (f_A), inflow water (f_I), water column (f_W), and sediment (f_S), we can write the fundamental mass balance equations for water and sediment compartments in the form:

$$\text{inventory change (mol/hr)} = \text{input rate (mol/hr)} - \text{output rate (mol/hr)} \quad (14.1)$$

TABLE 14.1

D Values Used to Describe the Equilibria and Kinetic Processes in the Mass Balance Model: Equations Used to Calculate These D Values, Process Rate Expressions D*f* (mol/hr), and Actual D Values for Anthracene and Pyrene Shown in the Illustrations

Process	Symbol	D Value (mol/Pa hr)	Process Rate (mol/hr)	Anthracene	Pyrene
Sediment burial	D_B	$G_B C_S$	$D_B f_S$	3.2	59.5
Sediment transformation	D_S	$V_S C_S k_S$	$D_S f_S$	57.0	330
Sediment resuspension	D_R	$G_R C_S$	$D_R f_S$	3.12	58.7
Sediment to water diffusion	D_T	$k_T A_S C_S / K_{SW}$	$D_T f_S$	101	435
Water to sediment diffusion	D_T	$k_T A_S C_W$	$D_T f_W$	101	435
Sediment deposition	D_D	$G_D C_P$	$D_D f_W$	41.9	787
Water transformation	D_W	$V_W C_W k_W$	$D_W f_W$	4840	7060
Volatilization	D_V	$k_V A_W C_W$	$D_V f_W$	348	389
Absorption	D_V	$k_V A_W C_A / K_{AW}$	$D_V f_A$	348	389
Water outflow	D_J	$G_J C_W$	$D_J f_W$	1580	6800
Suspended particle outflow	D_Y	$G_Y C_P$	$D_Y f_W$	22.4	422
Rain dissolution	D_M	$G_M C_A / K_{AW}$	$D_M f_A$	24.2	104
Wet particle deposition	D_C	$G_C C_Q$	$D_C f_A$	23.8	157
Dry particle deposition	D_Q	$G_Q C_Q$	$D_Q f_A$	8.9	58.8
Water inflow	D_I	$G_I C_I$	$D_I f_I$	1580	6800.8
Suspended particle outflow	D_X	$G_X C_X$	$D_X f_I$	108	2020

This equation is conceptually identical to that for money in a bank. The change in the balance over a month (the inventory change in $/month) equals the difference between the rate of deposits or inputs and the rate of withdrawals or outputs, also expressed in $/month. Mathematically, this is expressed by a differential mass balance equation for water and sediments. The fugacity versions are more concise than the concentration versions and are given in Equation 14.2 and Equation 14.3 to illustrate the concept.

$$\begin{aligned} d\left(V_W Z_W f_W\right)/dt = {} & E_W + f_I\left(D_I + D_X\right) + f_A\left(D_V + D_Q + D_C + D_M\right) \\ & + f_S\left(D_R + D_T\right) - f_W\left(D_V + D_W + D_J + D_Y + D_D + D_T\right) \end{aligned} \tag{14.2}$$

$$d\left(V_S Z_S f_S\right)/dt = f_W\left(D_D + D_T\right) - f_S\left(D_R + D_T + D_D + D_B\right) \tag{14.3}$$

Each of the D*f* terms represents the rate of a specific process (mol/hr or g/hr) by which the chemical enters, leaves, or changes location in the system. The groups (V*Zf*) are the total amount (mol or g) in the compartment. Values of each D must be established from environmental conditions and chemical properties. We make no attempt here to explain all such terms in detail; the aim is merely to convey that this is one approach by which a process mass balance can be established for a two-compartment system.

At steady state, which can be achieved after a long time with constant inputs, the water and sediment concentrations and fugacities become constant, the derivatives on the left become zero, and the two resulting algebraic equations can be solved to give the prevailing steady-state fugacities or concentrations:

$$\begin{aligned} f_W &= \frac{E_W + f_I\left(D_I + D_X\right) + f_A\left(D_V + D_Q + D_C + D_M\right)}{D_V + D_W + D_J + D_Y + \left(D_D + D_T\right)\left(D_S + D_B\right)/\left(D_R + D_T + D_S + D_B\right)} \\ f_S &= \frac{f_W\left(D_D + D_T\right)}{D_R + D_T + D_S + D_B} \end{aligned} \tag{14.4}$$

The differential equations can, if desired, be solved analytically or numerically, yielding equations that describe how the concentrations change with time (i.e., the dynamic situation).

TABLE 14.2

Input Data Used in the QWASI Model for the Hypothetical Lake

Property	Value
Water area (m^2)	10^6
Water depth (m)	15
Water volume (m^3)	15×10^6
Water residence time (days)	100
Sediment area (m^2)	10^6
Sediment active depth (m)	0.03 (3 cm)
Sediment volume (m^3)	30,000
Chemical concentration in air (ng/m^3)	100
Chemical concentration in inflow water (ng/L; $\mu g/m^3$)	1000
Water inflow rate (m^3/d; m^3/hr)	150,000; 6250
Direct discharge of chemical to water (kg/yr)	100

In summary, the key decisions and tasks of the modeler are as follows:

- Determine how many boxes, or compartments, must be included and obtain information on their dimensions and properties.
- Determine the relevant partitioning and reactivity properties of the chemical.
- Identify all the significant processes to which the chemical is subjected, including discharges and transport into the system (e.g., from the atmosphere).
- Write mass balance equations (differential or algebraic) for each box in terms of an unknown concentration or fugacity, including expressions for all process rates.
- Obtain all required parameter values for the system, including characteristics of the lake and chemical emission or discharge rates.
- Solve the differential or algebraic equations to obtain estimates of the unknown concentrations or fugacities.
- Calculate all masses of chemical and all input and output rates to ensure that they balance.

A complete mass balance is now available that can be inspected to determine the most important processes. The results can be compared with available monitoring data, and, if desired, a sensitivity analyses can be done to test the effect of varying the input data. The modeler now has a complete prediction or characterization of the chemical fate in the system subject, of course, to the inherent uncertainties of the model. The results can be assessed and used to guide remedial actions as appropriate. This should be done with an appreciation of the uncertainties.

Illustration of a Mass Balance Model in a Sediment–Water System

These concepts are best conveyed to the reader by an illustrative mass balance calculation. The situation addressed is a hypothetical one of a small lake of area 1 km^2 and average depth 15 m. The rates of water inflow and outflow are such that the water residence time is 100 days. The active layer of lake sediments is considered to be 3 cm deep. Typical values are assumed here for the parameters controlling air–water and sediment–water exchanges as listed in Table 14.2, and the actual D values used are listed in Table 14.1. Examination of the magnitude of these D values reveals the relative importance of the various processes. In practice, these quantities are all subject to uncertainty.

The fates of two chemicals in this lake are examined: the polycyclic aromatic hydrocarbons (PAHs) anthracene and pyrene with the properties listed in Table 14.3. These chemicals enter the lake from three sources: direct emission to the lake (possibly by runoff from contaminated soil or from an industrial operation), both chemicals are present in the inflow water at relatively low concentrations, and both are

TABLE 14.3

Physical/Chemical Properties of the Two Polycyclic Aromatic Hydrocarbons (Anthracene and Pyrene) Used in the Model Simulations

Property	Anthracene	Pyrene
Molar mass (g/mol)	178.2	202.3
Melting point (°C)	216.2	156
Vapor pressure (solid) (Pa)	0.001	0.0006
Water solubility (g/m^3)	0.045	0.132
Log K_{OW} (octanol–water partition coefficient)	4.54	5.18
Henry's law constant (H) (Pa m^3/mol)	3.96	0.92
Half-life in air (hr)	55	55
Half-life in water (hr)	550	1700
Half-life in sediment (hr)	17,000	55,000
Metabolism half-life in fish (hr)	720	1080

Note: The properties are taken from Mackay and Callcott (1998), Mackay et. al. (1992), and Verschueren (1996).

deposited from the atmosphere in which they have a known concentration. As a result of the presence of these PAHs in the water, fish are exposed to the extent that there may be toxic effects, and their tissues may be sufficiently contaminated such that human or wildlife consumption is inadvisable.

The overall task is to compile the mass balance model for this system for both chemicals. If this can be done, it will help answer such questions as:

- What is the dominant source of each PAH to the system?
- What fractions of the chemical are dissolved and sorbed in the water column and are thus different in bioavailability?
- What are the important rates of loss of PAH from the system—evaporation, degrading reactions, or advective outflow?
- What are the relative masses of the PAHs in the water column and the sediment?
- If levels in fish or water are judged to be a factor of (say) 10 too high, what is the best strategy for reducing inputs to achieve this goal?
- If such a loading (input rate) reduction is implemented, how long will it take for the system to recover?

Just as a corporate accountant requires a full understanding of cash flows to and from a corporation as a basis for effective management, the environmental scientist requires corresponding information on chemical flows so effective and economic remedial strategies can be implemented.

The lake mass balance model can be constructed in a variety of ways, usually with the aid of computer programs that reduce the tedium of the calculations. In principle, all programs should give the same results; however, minor differences in how the various processes are treated usually produce somewhat different results. The model used here is the steady-state Quantitative Water Air Sediment Interaction (QWASI) model, which has been described by Mackay (2001) and is available for general use (www.trentu.ca/cemc). The QWASI model uses the steady-state equations shown earlier. In this model, all D values are estimated from data or parameter values obtained on the properties of the chemicals and the lake. Here, we focus on the input and output data and not the detail of the calculations. The model is of two well-mixed boxes: the water column and the accessible bottom sediment. A total of 17 input and output processes must be quantified. The essential strategy for reaching the solution, as was described earlier, is to write down the mass balance equations for the water and sediment boxes and to express all rates in terms of the unknown concentrations in each box. This gives the two mass balance equations presented earlier containing two unknowns, which can thus be solved. All process rates can be deduced and checked to ensure that the total inputs are equal to the total outputs, the condition that must apply at steady state. Some unsteady-state or dynamic aspects are discussed later. Table 14.2 and

TABLE 14.4

Selected Output Data from the QWASI Model Simulation of Chemical Fate in the Hypothetical Lake

	Anthracene	Pyrene
Fugacities (Pa)		
Water column	1.46×10^{-05}	5.8×10^{-06}
Sediment	1.27×10^{-05}	8.02×10^{-06}
Fluxes (kg/year)		
Sediment burial	0.063	0.845
Sediment transformation	1.128	4.693
Water transformation	110.109	72.558
Volatilization	7.916	3.998
Water outflow	35.901	69.830
Water particle outflow	0.510	4.333
Total water and particle outflow	36.411	74.163
Concentrations		
Air concentration (ng/m^3)	100	100
Total water concentration (ng/L)	665.0	1354.6
Total sediment concentration (mol/m^3)	17.7	210.2
Total concentration in particles (ng/g)	1864.4	15,829.7
Total concentration in sediment solids (ng/g)	291.1	3932.7
Total concentration in sediment pore water (ng/L)	570.3	1765.3
Mass		
Water (kg)	9.98	20.32
Sediment (kg)	3.16	42.52
Total mass in system (kg)	13.13	62.84

Table 14.3 give the input data for these chemicals, including the physicochemical properties of the two PAHs and the physical parameters of the illustrative lake. Table 14.4 gives selected output data, including concentrations (g/m^3), fluxes (kg/year), and masses of the PAHs in water and sediment. Figure 14.2 and Figure 14.3 present these data pictorially.

Anthracene

Figure 14.2 shows that of the total anthracene input of 155.6 kg/yr, most (100 kg/yr, or 64.2%) enters the system by direct discharges with water inflow contributing 54.7 kg/yr (35.2%) and atmospheric absorption and deposition 0.876 kg/yr (0.56%). The water column contains 9.98 kg at a concentration of 0.000665 g/m^3 or equivalently 665 $\mu g/m^3$ which is sufficient to generate loss processes that dissipate the inputs exactly. There is a total transfer of 3.25 kg/yr to the sediment, but 2.06 kg/yr returns by resuspension and diffusion; thus, there is a net transfer of 1.19 kg/yr. Other losses in decreasing order of importance are reaction in water, 110 kg/yr (70.8%); outflow, 36.4 kg/yr (23.4%); and evaporation, 7.9 kg/yr (5.1%). Of this net transfer of 1.19 kg/year from the water column to the sediment, most is degraded (1.128 kg/yr), with some (0.063 kg/yr) being permanently buried in inaccessible sediment. The mass in the sediment is 3.16 kg, about one third the mass in the water column. Of the total 665 $\mu g/m^3$ in the water, 656 $\mu g/m^3$ is in solution (and may thus be bioavailable) and 9 $\mu g/m^3$ is sorbed to suspended sediment; thus, all but 1.3% is in solution. This is typical for a relatively low suspended solids concentration such as 5 mg/L. If this was 20 mg/L, the percent sorbed would increase to about 5%.

Key additional quantities that emerge from these results are the residence times of the chemical in each compartment. The residence time is defined as the mass in the compartment divided by the total input or output rate. It can be viewed as the average time the chemical resides in the compartment before being removed. For the sediment, this is 3.16 kg divided by 3.251 kg/yr, or 0.97 year. For the water column, it is 9.98 kg divided by 155.6 kg/yr, or a shorter 0.064 year (23 days). Overall, for the entire system, the residence time is 13.13 kg divided by 155.6 kg/yr, or 0.084 year (31 days). The implication

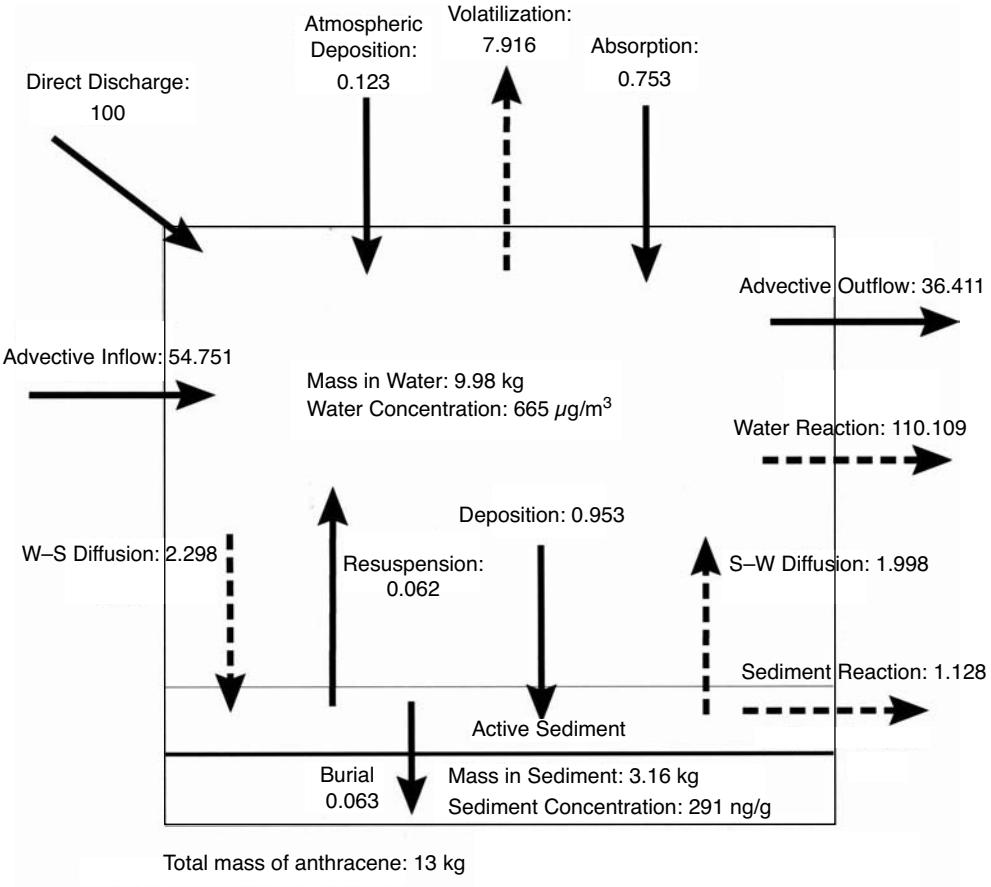

FIGURE 14.2 Process rates of the chemical fate of anthracene in the hypothetic lake environment (expressed as kg/year).

is that if a change is made to inputs, the water column will respond fairly rapidly, but the sediment will respond much more slowly. After 2.3 residence times, a system should be 90% toward its new steady-state condition. In reality, the water column will respond rapidly, the sediments more slowly; also, some slow bleeding from sediments back to the water column will retard the full response of the water. The longer response time of the sediment will be the ultimate controlling factor. A conservative estimate is that the abiotic system will be 90% on the way to recovery some 7 years after the direct inputs are reduced.

Inspection of the mass balance equations shows that to reduce concentrations by a factor of 10 simply requires reducing total inputs by this factor to about 16 kg/year. In this case, a feasible strategy is perhaps to assume that atmospheric inputs remain constant and to reduce the advective inflow rate of anthracene by 80% to about 11 kg/yr and discharges by 95% to 5 kg/yr, in the expectation that after some 7 years the new target levels will be approached.

Pyrene

For pyrene, the general picture is similar (Figure 14.3) but there are some notable differences of detail. Pyrene, being more hydrophobic, has a higher octanol–water partition coefficient, so it sorbs more strongly to suspended and bottom sediments. Further, it is less volatile and less reactive. The result is that, although the inputs are similar to those of anthracene, the mass in the system is larger (i.e., 20.32 kg in the water column and 42.52 kg in the sediment). The overall residence time is thus 0.4 years, or 5 times longer. Pyrene is less bioavailable in the water, as 94% is in solution compared with 99% for anthracene. It is thus important to appreciate that the fate of a chemical in an aquatic system depends

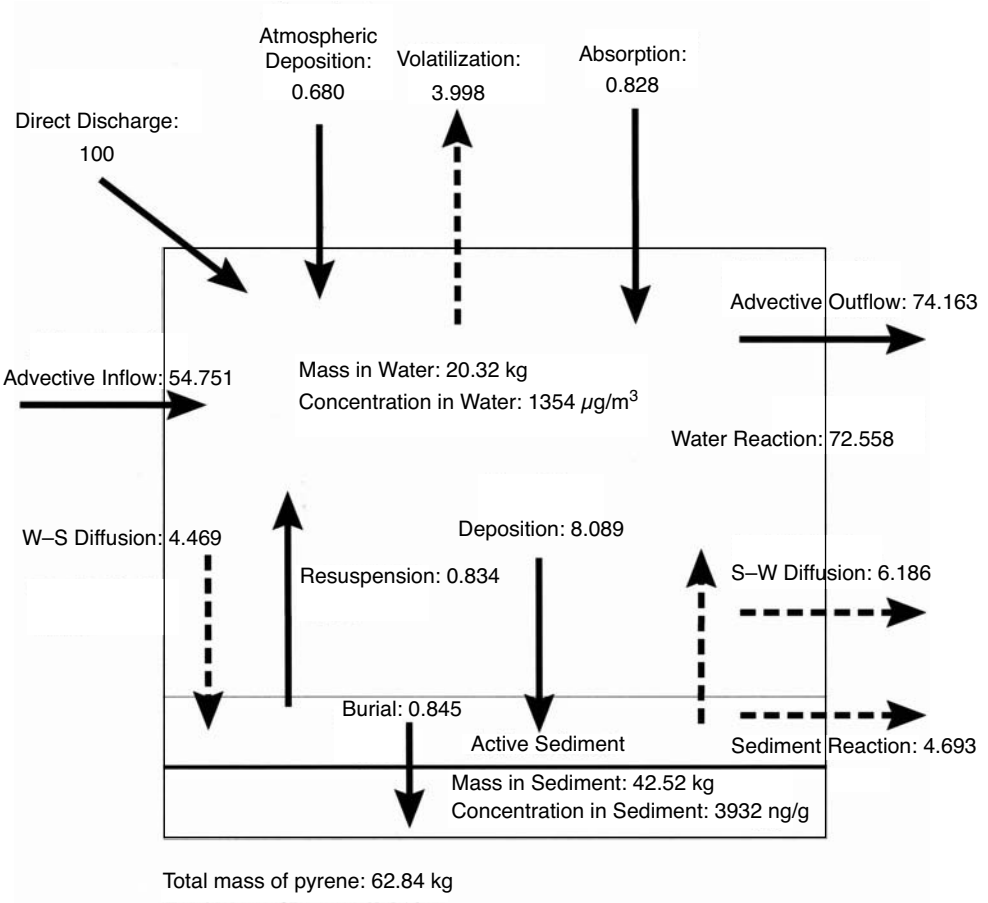

FIGURE 14.3 Process rates of the chemical fate of pyrene in the hypothetical lake environment (expressed as kg/year).

on both the properties of the chemical and those of the environment in which it is distributed. Chemical properties can vary over a range of 10^{12}, so the fate of a very volatile chemical such as chloroform is entirely different from that of the involatile benzo(*a*)pyrene. Only with the aid of a model can these differences be assessed, evaluated, and predicted in advance.

Bioaccumulation

Chapters 2 and 3 gave comprehensive reviews of bioavailability and bioaccumulation, so no attempt is made here to repeat this material; however, it is useful to demonstrate how the abiotic fate data presented above can be used to calculate concentrations in fish. This can be accomplished by one of several models in which the box receiving inputs and generating outputs is a fish (Figure 14.4). In more sophisticated pharmacokinetic models, the fish can be treated as a series of connected boxes, each box corresponding to an organ or a group of tissues. To extend the illustration, we apply the FISH mass balance model (www.trentu.ca/cemc) to both chemicals, using the water column concentrations previously determined. The fish considered is a 10 g trout as specified in Table 14.5. The output data and fluxes are given in Table 14.6 and Table 14.7.

For anthracene, the whole body wet weight concentration is 1.14 g/m^3 or μg/g. Uptake is 21.8% from food and 78.2% from respired water for a total input rate of 1.04 μg/day. The bioconcentration factor expressed as the ratio of concentration in fish to that dissolved in water is 1713. The losses are 0.176

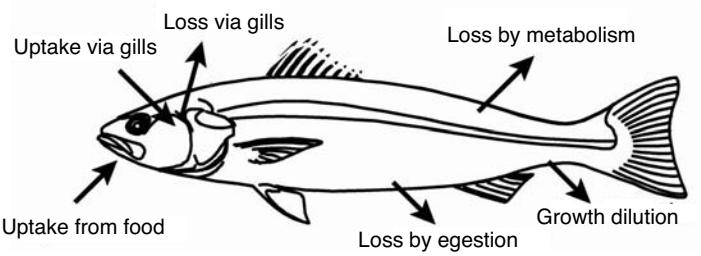

FIGURE 14.4 Uptake and loss processes of a fish for organic chemicals.

TABLE 14.5

Properties of the Fish Used for the Model Simulation

Fish Property	Value
Fish volume (cm^3; m^3)	10; 0.00001
Fish lipid volume fraction	0.05
Food lipid volume fraction	0.05
Gill water resistance time (hours)	0.001
Gill organic resistance time (hours)	300
Gut absorption water resistance	1.00E–07
Gut absorption organic resistance	2
Digestion factor	3
Growth rate as a fraction of volume per day	0.001
Feeding rate as a percent of body weight per day	2

TABLE 14.6

Results from the Fish Model Simulation

Property	Anthracene	Pyrene
Concentration in fish (whole body) (g/m^3)	1.14	9.67
Concentration in fish (lipid) (g/m^3)	22.8	193.5
Concentration in food (whole) (g/m^3)	2.27	19.3
Concentration in food (lipid) (g/m^3)	45.4	386
Bioconcentration factor	1713	7139
Fish/water fugacity ratio	1.00	1.00
Fish/food fugacity ratio	0.501	0.501

TABLE 14.7

Rates of Uptake and Clearance Process of Chemicals in the Fish at Steady State

Fluxes (μg/day)	Anthracene	Pyrene
Net uptake from food	0.23	1.92
Uptake through gills	0.81	5.13
Total net input	1.04	7.05
Loss through gills	0.82	5.14
Loss by egestion	0.04	0.32
Loss by metabolism	0.18	1.49
Loss by growth dilution	0.01	0.09
Total net output	1.04	7.05
Overall half-life (hours)	181.9	228.21
Overall half-life (days)	7.58	9.51

TABLE 14.8

Models of Aquatic Systems and Bioaccumulation

Model	Scope	Refs.
EXAMS	Exposure Analysis Modeling System	Burns (2002)
WASTOX	Water Quality Analysis Simulation of Toxics	Connolly and Winford (1984)
WASP	Water Quality Analysis Simulation Program	Ambrose (1998)
QWASI	Quantitative Water Air Sediment Interaction fugacity model for lakes	Mackay (1998, 2001)
ROUT	GIS model applied to U.S. rivers	Wang et al. (2000)
GREAT-ER	European GIS river basin model	Feijtel et al. (1997)
DITORO	Sediment water exchange	Di Toro (2001)
GOBAS	Model of fish and food webs	Gobas (1993)
AQUATOX	Aquatic fate toxicity model	Park (1998)
FGETS	Food and Gill Exchange of Toxic Substances	Barber (1991)
FISH & FOODWEB	Fugacity model of fish and aquatic food web	Mackay (2001), Campfens and Mackay (1997), Canadian Environmental Modeling Centre (www.trentu.ca/cemc)
THOMANN	Model of fish and food web	Thomann (1989)

μg/day by metabolism, 0.038 μg/day by egestion of feces, 0.817 μg/day by respiration, and 0.011 μg/day by growth dilution. The residence time in the fish is thus approximately 7 days. The quantities given earlier are obviously only as accurate as the input data. In practice, there is often considerable uncertainty about quantities such as metabolic rates, feeding rates, and lipid contents. In this case, it is probable that the uncertainty in concentrations, masses, and fluxes is about a factor of 3. This can be estimated by adjusting the parameters and exploring the effect on the output quantities. One approach is to test each parameter individually (e.g., doubling and halving the degradation rate constant). A more complete assessment would involve a Monte Carlo analysis in which all parameters are varied simultaneously within prescribed limits, and by repeated simulation (say, 1000 times) a distribution of output quantities is obtained. For pyrene, the bioconcentration factor is higher at 7139 because of its greater hydrophobicity. This bioconcentration factor is in reasonable agreement with those found by de Voogt et. al. (1991) in guppies (*Poecilia reticulata*). The rate of input of pyrene to a fish is approximately 7 times higher than for anthracene.

A variety of models is available, all of which should, in principle, give similar results. The key conclusion is that the availability of fate and bioaccumulation models offers the potential to translate information on inputs to lakes or rivers into estimates of fish concentrations and body burdens. Table 14.8 lists a selection of models available for assessing chemical fate in aquatic systems and uptake by organisms. A review by Paquin et al. (2003) describes exposure, bioaccumulation, and toxicity data for metals, but many models can also be applied to organics. A useful source of U.S. EPA models is www.epa.gov/osp/crem.htm.

Discussion and Conclusions

The models presented here are relatively simple. In practice, it is often desirable to segment a lake, river, or estuary into a number of connected boxes. Segmentation can be on a horizontal or vertical basis, or both. The greater the number of segments, the more accurately the model can describe spatial concentration differences, but this does not necessarily improve the overall accuracy of the predictions. Unfortunately, this improved spatial resolution is obtained at the expense of a more complex model that demands more input data. The art of modeling lies, in large part, on the selection of the optimal number and arrangement of the segments or boxes. The segmentation of the Bay of Quinte (Lake Ontario) to assess the fate of several chemicals offers some insight into the number and arrangement of boxes required to characterize that particular system (Diamond et. al., 1996). Similar principles apply to biotic models in which the fish can be treated as a series of internally connected compartments. Such

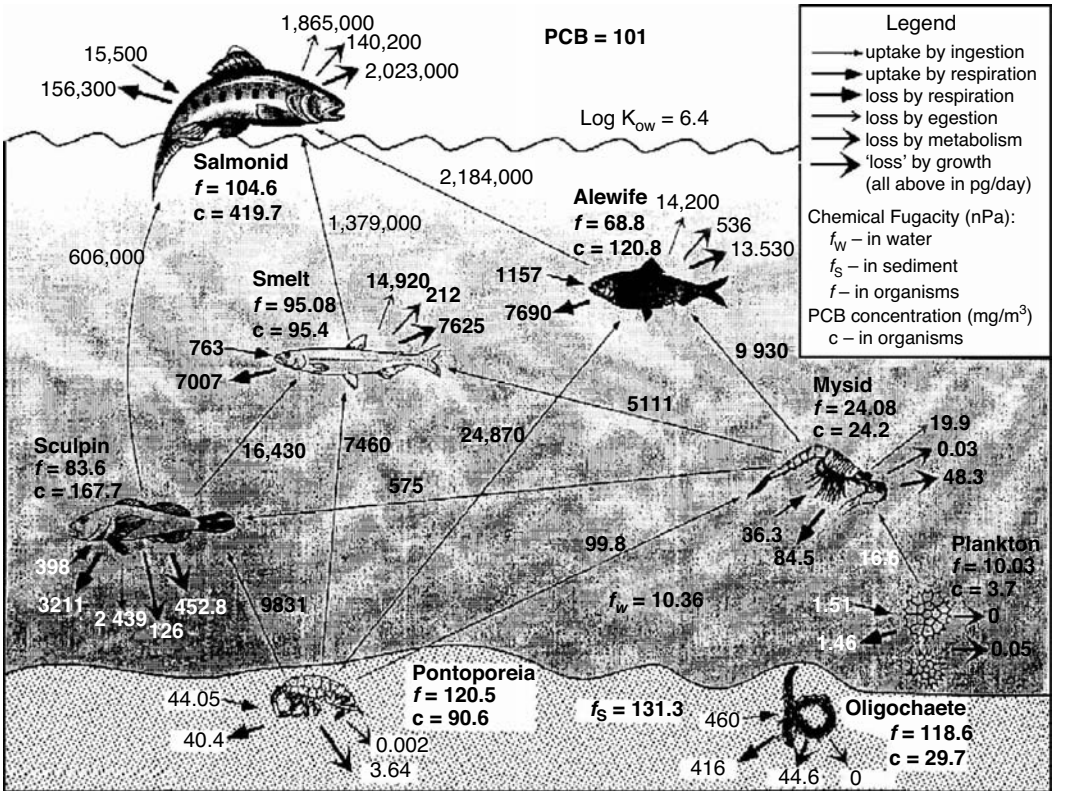

FIGURE 14.5 An example of a complex food web model (Lake Ontario food web); the concentrations, fugacities, and fluxes (μg/day) for PCB congener 101 are shown. (From Campfens, J. and Mackay, D., *Environ. Sci. Technol.*, 31, 577–583, 1997. With permission.)

physiologically based pharmacokinetic (PBPK) models are invaluable when exploring how chemicals become distributed in fish. Examples are the models of Gobas et al. (1993). It is also possible to build models of food chains or food webs in which a variety of organisms feed on each other transferring contaminant by ingestion. Figure 14.5 is an example of an attempt to model the fate of a PCB congener in the complex Lake Ontario food web (Campfens and Mackay, 1997).

This chapter has sought to convey the principles underlying mass balance modeling in both the abiotic and biotic spheres and has provided a glimpse into the benefits derived from obtaining a full quantitative understanding of contaminant fate in aquatic systems. Again, the complementary nature of monitoring efforts and modeling tools is emphasized. Both can contribute, along with laboratory investigations, to a fuller understanding of how chemical substances can impact fish and other aquatic organisms.

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15

Fish Toxicity Studies

Gary M. Rand

CONTENTS

Background/History

Fish are ecologically and economically important. They represent a group of vertebrates with diverse behavioral and reproductive strategies and play a significant role in the food chain as consumers (predators) and the consumed (prey). During their life cycle, most fish also feed on a broad range of items. Although fish may not always be the most sensitive aquatic organisms to chemical stressors, they certainly have a wide range of behaviors and habits that increase their potential for exposure to chemicals in different environmental matrices (e.g., dissolved, adsorbed, suspended, deposited). Furthermore, extensive literature exists on their behavior, physiology, and general environmental requirements (Evans, 1993; Hoar and Randall, 1969–1988; Hoar et al., 1992–2001). Fish thus constitute a relevant group of test organisms to evaluate the biological effects of toxic chemicals.

General test methods used by fish toxicologists have their origin from the 1800s (Penny and Adams, 1863) and were adapted from general techniques used in mammalian toxicology. Goldfish and minnows were the first fish species used in aquatic toxicity tests to determine the effects of chemicals used in dye-works (Penny and Adams, 1863), but it was not until the early 1900s that fish toxicity testing became

a reality. Laboratory and field (*in situ* cage studies) investigations were conducted with lead to assess the impact of mine tailing effluents (Carpenter, 1925, 1930). Carpenter was also the first to note the effects of hardness on the toxicities of divalent metals. Belding (1927) and Ellis (1937) described the conditions (i.e., test variables) that affected fish as test organisms and dilution water quality. Ellis also established one of the first hazard ranking systems based on fish responses to municipal and industrial effluents. Prior to World War II, most toxicity studies with fish (e.g., goldfish, minnows, salmonids) were conducted with regard to industrial wastes and metals, and some early attempts were made to develop and describe standard acute toxicity tests with fish. It was not until after World War II, however, that efforts were directed toward standardizing techniques for fish acute toxicity tests (Hart et al., 1945). It was the era of the "pickle-jar bioassays," in which acute tests with single species were conducted in 5-gallon pickle jars. Scientists from Canada, the United States, and England demonstrated the utility of exposing fish to industrial wastes and other chemicals for predicting the potential toxicity to freshwater systems. Fish tests were also used to assess the effects of environmental factors (e.g., changes in temperature, dissolved oxygen) on toxic responses. Work by the earlier groups of scientists established acute toxicity tests as the workhorse for studying and monitoring pollution effects (Buikema et al., 1982), a position it still retains. The fish acute toxicity test was the basis for the first aquatic toxicity protocol of the American Society for Testing and Materials (ASTM), and, in the 1960s, the American Public Health Association included fish toxicity test procedures in its well-established *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 1960).

Interest and concerns about chronic, low-level exposures occurred during the 1960s as a result of the distribution of organochlorine compounds. This stimulated the development of flow-through techniques for use in studies on long-term exposures of aquatic organisms to chemicals. Experiments were also initiated in the 1960s that included exposures during early life stages and full life cycles, as well as the measurement of sublethal endpoints. The 1970s also saw acute fish toxicity being used as an acceptable parameter for protocols used in regulatory testing. In the 1980s and 1990s, there was a greater appreciation for understanding mechanisms of toxic action (or modes of action; see Chapter 5) of chemicals and suborganismal levels of assessment (see Chapters 7 to 13). Without knowledge of the sites of action (or biochemical lesions), it is possible that potentially important physiological effects may be undetected and overlooked. Greater use of different fish toxicity techniques were built into regulatory guidelines, regulations, and codes of practice throughout the world. For a good summary of the history of acute toxicity testing with fish, see Hunn (1989). Also, for a summary of fish toxicity testing techniques, see Sprague (1969, 1970, 1971).

It is important to study other aquatic organisms and different levels of organization in aquatic systems because of the roles different organisms play in food chains; however, this chapter focuses on laboratory fish toxicity tests. Toxicity tests are traditionally conducted to assess or predict the biological effects of chemicals (e.g., industrial, pesticides, PAHs) and determine the relative toxicity of such substances. Just as there are many different biological levels of organization at which a chemical can exert effects, a variety of toxicity test methods are available to investigate the type and severity of such effects. General concepts and test methods for fish are presented in this chapter, although they can be applied to other species.

Fish toxicity tests can be conducted in the laboratory or in the field, and the individual organism is the basic unit of study. Laboratory tests with fish have been standardized with established protocols and greater control over environmental variables then field tests (see Chapters 19 to 25). Few standardized protocols are available for field tests with fish and other aquatic organisms. Historically, most fish tests have been done in the laboratory, and most hazard and risk evaluations used in risk management decisions are made on the basis of laboratory results. Field validation of laboratory-derived toxicity tests, however, is often considered a necessary tool in the ecological risk evaluation process (see Chapter 18).

Objectives and Limitations

Toxicity tests may be used to meet one or more of the following objectives:

- To develop data to better understand biological effects and exposure
- To generate data to support decisions for risk assessment and risk management

- To meet regulatory requirements to regulate development, manufacture, or release
- To determine chemical classifications (and rankings)
- To develop numerical water and sediment quality criteria

The toxicity test method and design selected depend on the specific questions to be addressed. Toxicity test programs usually focus on one or more of the following questions:

- Is the substance lethal to test organisms and at what concentration?
- What are the effects on organisms exposed to low, sublethal concentrations during part or all of its life cycle?
- What are the effects of short-term "slug" or "spike" exposures (or episodic events)?
- Which chemical is most toxic?
- What indigenous organism is most sensitive, and what is the distribution of sensitivities?
- What are the environmental conditions that make the chemical most toxic? Interaction with environmental variables?
- Is the toxicity of the chemical similar in laboratory and field?
- What are realistic environmental exposure concentrations, and what is the safe concentration of the chemical?
- What are the effects of mixtures of chemicals?

Laboratory toxicity tests with juvenile fish are typically conducted with single-species exposures, and most often only a few standard fish species are studied for a single chemical in an aquatic toxicity program. The results of a limited number of standard test species are often used to extrapolate to a vast number of indigenous species making up an aquatic community. Indigenous species are most often used in toxicity testing on a site-specific basis when testing effluent discharges, when potential hazards are predicted, or in monitoring programs. Indigenous species are typically not used in testing industrial chemicals or pesticides. Aquatic toxicity programs, based on their objectives, should incorporate the use of multiple, indigenous fish species from a range of behavioral strategies.

Identifying the toxicological effects of a chemical on individuals in the laboratory are most often easier to determine than the effects on communities because in nature effects are regulated by the physicochemical conditions of systems that can enhance or inhibit toxic impacts on organisms. Chemicals can act in concert with other chemical and nonchemical stressors. Organisms in natural systems may also be physiologically or immunologically compromised prior to chemical exposure. This is common today in many freshwater and saltwater systems throughout the world because of prior widescale contamination of organochlorine compounds, such as dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD). Furthermore, species may differ in the uptake, accumulation, distribution, metabolism, and excretion of chemicals (see Chapters 2 to 4). Species differences are another reason why extrapolation to communities is difficult. Our ability to predict ecotoxicological effects from single species tests is rudimentary; however, our potential to predict ecotoxicological effects on communities will be expanded when studies evaluate realistic interactive effects. Another approach in testing is to study the effects of realistic environmental exposure concentrations and then define the range of the lowest exposure concentrations that produce adverse responses for indigenous species and examine the extent to which environmental conditions alter minimum exposures and different responses.

Single-species fish tests are technically an initial step to study the direct effects of single chemicals, effluents, and mixtures of chemicals. *Direct* toxic effects on individual fish refer to lethality and to biochemical, physiological, and behavioral changes that may result from exposure and affect function. *Indirect* (or *secondary*) effects are more complex to assess. Indirect effects are those effects that occur as a result of a direct effect on the reduction or elimination of susceptible species (prey or predator) and may include disturbances in biological interactions and processes. Indirect effects may also occur as a result of a direct effect on the physical or chemical environment; for example, a chemical that decreases predator abundances may increase prey abundance and competition. This result may be classified as a

TABLE 15.1

Acute Test Objectives

Test effects of high exposure concentrations of short duration.
Determine intrinsic and relative toxicity (ranking) and lethal concentrations (LC_{50} values).
Set concentration ranges for long-term studies.
Provide initial hazard data for chemical labeling.
Determine mechanism of toxicity.
Determine effects of modifying factors (e.g., temperature, pH, salinity) on toxicity.

direct effect on the predator and an indirect effect on the prey species. Indirect responses (or effects) on aquatic organisms have been studied in multispecies model systems such as microcosms and mesocosms because they provide temporal and spatial variation of organisms and chemicals exemplified and more consistent in the natural environment; thus, they permit the components of a system to interact and allow direct and indirect effects to occur. Multispecies tests use several species from one trophic level or many species from different trophic levels. Single-species tests do not display the latter characteristics and have limited extrapolation potential. Recently, however, Versteeg et al. (1999) summarized chronic single-species toxicity data on a variety of chemicals, including heavy metals (cadmium, copper, zinc), pesticides (atrazine, lindane), surfactants (C12 LAS), two organic substances (phenol and 3,4-dichloroaniline), and two inorganic substances (ammonia and chlorine). Analyses suggested that a sufficiently large dataset of laboratory-generated, chronic, single-species data can be used to set concentrations protective of model ecosystems and likely whole ecosystems. No evidence suggests, however, that the same relationship occurs for acute tests and whole ecosystems.

Types of Tests

Laboratory tests with fish can be classified by the duration of the exposure period and measured endpoints. Tests are categorized as acute, subchronic, and chronic as a result of exposure time relative to the length of the organism's life cycle. For fish, acute tests are conducted for periods up to 96 hours; however, for a vertebrate with a short life cycle (6 to 9 months), tests conducted with exposures for several weeks may also be considered acute tests, as exposure to the chemical is still only a fraction of the life cycle. Objectives of acute tests are listed in Table 15.1. The fact that a chemical does not have adverse effects on fish from short-term exposures does not indicate or imply that it is not toxic. Alternatively, a chemical that does elicit a rapid response, such as death, does not imply that it is not likely to elicit effects at lower concentrations. Furthermore, for chemicals that have low water solubilities and high bioconcentration potential, short-term exposures may not be adequate to assess biological effects.

Subchronic exposure tests are less than an entire reproductive life cycle and usually consist of repeated (continuous or intermittent) exposures to low, sublethal levels of a chemical. For fish, subchronic studies have been traditionally called *early life stage* (ELS) tests because they evaluate adverse effects on critical early life stages (i.e., egg, embryo, larva, fry). Sublethal subchronic exposures with fish involve continuous exposure to the chemical from newly fertilized embryos through early fry stages and are usually 1 to 3 months. For example, subchronic exposure tests with warmwater fish are usually 1 month in duration, and those with coldwater fish are usually 2 to 3 months. Tests are conducted until at least 30 days after swim-up with coldwater fish. Alternatively, we could also expose fish for only part (e.g., weeks to months) of the adult stage and assess effects on reproduction; because of the duration of exposure, this type of test may also be considered a subchronic one. Objectives of subchronic (ELS) studies are listed in Table 15.2.

Chronic (i.e., long-term) exposure tests with fish may further consist of two types of exposures based on duration and are thus called partial or full life-cycle tests. They usually begin with exposing newly spawned eggs, newly hatched larvae, juveniles, or sexually immature fish and continue through sexual maturity and reproduction (i.e., spawning). The initial life stage selected for exposure depends on the species, availability of stages, and test objectives. These exposure tests are used to evaluate lethal and

TABLE 15.2

Subchronic Test Objectives

Identify effects not detected in short-term acute tests that maybe triggered by longer exposures.
Provide additional data on effects identified in acute tests.
Identify lowest-observed-effect concentrations (LOECs) and no-observed-effect concentrations (NOECs) for biological endpoints measured.
Provide information for concentration selection and study design features for longer-term studies.
Identify sensitive life stages (eggs, larvae, juveniles) and endpoints (hatching time, survival of fry, growth, deformities).
Provide basis for species selection for additional studies.
Provide data for regulatory agencies in support of risk assessments.

sublethal endpoints, but they are primarily used to look at how long-term, low-level chemical exposure of an initial F_0 generation affects reproduction and the F_1 generation (viability) after 30 to 60 days after hatching. Effects (endpoints) on reproduction are measured by the number of spawns and the number of eggs per spawn (fecundity) following exposure of the entire life cycle of the species. Partial life-cycle (or partial chronic) exposure tests are conducted with fish species that require more than 1 year to attain sexual maturity (e.g., trout, bluegill), and they may be 12 to 15 months in duration. These tests may also be classified as subchronic tests because exposures are less than a full life cycle. Full life-cycle tests with freshwater and saltwater minnows have been 6 to 9 months long.

Basic Concepts

Fish toxicity tests are conducted under controlled water-quality and environmental conditions. The main routes of chemical uptake for fish include direct uptake from water (across gills and skin), food (across the alimentary canal), and sediment (ingestion of sediment or infaunal organisms across the alimentary canal) (see Chapter 2). The relative importance of exposure routes may differ between fish species and between chemicals and may be dependent on a multitude of environmental conditions (e.g., humic substances) in the wild. Most fish toxicity testing has focused on chemical exposures in water. *Concentration* is the term used to define the measured quantity of substances in a specific volume of water, sediment, or other material. The units of concentration depend, in part, on the medium and on the process being measured. It is obviously critical that units of concentration be clearly understood. In water, the common expression of concentration is mass of chemical per unit volume of water. The concentration of test chemical in water is expressed in parts per million (ppm) or units of test chemical per 10^6 units of dilution water (diluent). The common ratio is milligrams (mg) of test chemical per liter (L) of diluent water. The concentration may also be expressed in parts per billion (ppb) or units of test chemical per 10^9 units of diluent water. Here the ratio is micrograms (μg) of test chemical per liter (L) of diluent water. If fish are exposed to solutions of liquid wastes of an effluent, the concentration of effluent in water is expressed as volume % (100 × volume efficiency/volume effluent + volume dilution water). Expressions of chemical concentrations in the media, such as sediment or tissue, are in parts per million (or milligrams of test chemical per kilogram of tissue or sediment; 1 ppm = 1 mg/kg). Parts per billion correspond to micrograms (μg) of test chemical per kilogram (kg) of tissue or sediment or nanograms (ng) per gram (g). Weight-specific concentrations in sediment or tissue may be expressed as wet or dry weight. Molarity refers to number of moles per liter of solution and is denoted as m/L or mol/kg. From a toxicity perspective, the use of molar concentrations is extremely relevant because effects resulting from exposure to chemicals are usually a function of molecules present at target sites, not the mass of these molecules. Toxicity test results should be expressed in molar units.

Effects from exposures may range from rapid death to sublethal effects to no effects at all. Also, important and very relevant effects (e.g., changes in behavior, enzyme, and hormone levels) may be overlooked in toxicity testing. Effects may occur during exposure or after termination of exposure to a chemical, or they may be delayed until some time after termination of exposure. The latter types of

effects are a result of the properties of the chemical, mode of action of the chemical, and metabolic competency of the organism to change the form of the chemical. Some effects are reversible in that damaged tissue may be repaired or recovery may occur following narcosis (i.e., nonselective toxicity). A distinction in toxicological effects may also be made on the basis of site of action of a chemical (Chapter 5). Local effects occur at the primary site of chemical contact (e.g., gill inflammation). In contrast, systemic effects of a chemical require absorption and transport in the blood to a site distant from the contact or entry site.

Certain chemicals may produce nonselective toxicity as a result of general disruption of different cells and membranes. Narcosis is a baseline or minimum effect that every organic chemical can produce except that it may be masked by a more specific effect acting at lower concentrations. In essence, fewer molecules are required to cause the specific toxic response than is the case for narcosis. If a chemical has specific toxic activity, it is the mode of action that is expressed first. Chemicals that act on a specific tissue, enzyme, or process without harming others are selective. They usually produce effects at lower concentrations then chemicals that act through a nonselective mechanism. Rapid death is the most obvious and overt response (effect) to chemical exposure and is typically preceded by other premonitory signs of intoxication and malfunction (see Chapters 6 to 12). A major concern in fish toxicology, however, is the extent to which fish survive low-level exposure concentrations (sublethal) but function less efficiently because of less obvious signs of injury. In a sublethal toxicity test, the lowest-observed-effect concentration (LOEC) is the one that produces at least one statistically significant observed effect compared to control groups upon the most sensitive measured performance criteria (e.g., growth, reproduction). It is reasonable to assume that in the field any adverse biological effect that reduces the organism's ability to cope in the environment has a probability of impacting some aspect of its overall lifespan; however, sublethal effects may not reasonably be preliminary stages of death. Also of interest is the exposure concentration at which fish can survive, grow, and reproduce and show no adverse symptomology. In a toxicity test, the highest test concentration that produces no statistically significant adverse effect on the performance criteria is the no-observed-effect concentration (NOEC). The LOEC and NOEC are statistically defined concentrations in tests. The term *safe concentration* is a biologically defined concept.

The above implies that sublethal exposure to a chemical either exerts no effects if the exposure is below a toxic threshold concentration or has an adverse effect, the severity of which increases with increasing chemical concentrations. Note that, in toxicology, tests are used to study negative or adverse affects as a result of chemical exposure, but effects of chemical exposure may be stimulatory or positive deviations (e.g., increased growth) from background. There may also be an intermediate subinhibitory exposure concentration that is actually beneficial to organisms. Southam and Erhlich (1943) termed this phenomenon *hormesis* (after the Greek word for "to excite"). The organism, when challenged with low levels of stress, overcompensates and not only eliminates any low-level induced damage but also reduces background stress or damage more effectively than before the stress was applied (Calabrese and Baldwin, 2000). This response is independent of phyla, chemical class, and endpoint. The low-concentration (or low-dose) hormetic responses are not easy to differentiate from typical background variations. It is not known how frequently hormetic responses occur because toxicity tests are generally designed to deal with high concentration responses to determine NOECs. Hormetic responses imply the existence of significant biological activity below the traditional NOEC that typical toxicity testing cannot evaluate unless multiple concentrations are used in the low concentration range to determine the consistency and variability of stimulatory effects.

Concentration–Response Relationship

Toxicity tests are guided by the concentration–response relationship—that is, the relationship between exposure concentration (in water) or dose (in sediment, food) of a chemical and the response of the biological system (e.g., whole organism, cell) being exposed. The relationship has several assumptions:

- A cause-and-effect relationship exists, and there is a high degree of certainty that the response or effect is a result of organism exposure to the chemical being investigated.

- A concentration–response (or dose–response) relationship exists such that:
  - The response is a result of the chemical interacting with the site of action.
  - The quantity reaching the site of action is a function of the water, sediment, or food concentration to which the organism is exposed.
  - Above an effect-threshold concentration, the magnitude of effects will be proportional to the amount interacting at the site of action.
  - The effects of chemical exposure can be measured or quantified and are reproducible under a similar set of conditions.

Innocuous chemical substances can have undesired adverse effects when organisms are exposed to high concentrations over short time periods. Conversely, exposure to low concentrations of toxic chemicals may cause no apparent adverse effects. A significant factor in determining whether a chemical is potentially harmful or safe is the relationship between chemical exposure concentration and duration of exposure of the organism. Another relevant point is that the amount at the site of action is what is actually producing a biological effect (e.g., death, development), not the quantity in the water, sediment, or food. Quantities of chemical in media are only surrogates for actual concentrations of chemicals reaching sites of toxic action.

For a specific effect (or response), fish in a population respond differently to a stimulus because of inherent, natural biological variation reflecting the genetic makeup of the population and the condition of individuals. The relationship can be explained by a probability distribution for frequency of responses. Variation should be small for individuals of the same species and similar age and health but should generally be greater between species. Some individuals will be highly sensitive to a chemical, whereas others will be very resistant. When individual responses are distributed normally within the population (i.e., most individuals are neither sensitive nor resistant), a bell-shaped population curve results. This is the basic form of the concentration–response relationship. Note that many biological response distributions are not normal and tend to be skewed to high concentrations (i.e., resistants in populations have a greater range of responses to concentrations than the sensitive part of the population).

In measuring toxicity, the objective in a standard toxicity test is to estimate the range of exposure concentrations that produce selected quantifiable responses in groups of test species under controlled water quality and environmental conditions. Results of tests are plotted on a graph that relates the percentage of organisms in test groups that elicit defined responses (dependent variable, *y*-axis) to exposure concentrations of the test chemical (independent variable, *x*-axis). The concentration–response relationship developed from the plot is a graded relationship between concentrations of test chemical to which organisms are exposed and severity of response. It is fundamental to toxicology testing. In general, the greater the exposure concentration, the greater the response. The curve drawn to represent the relationship will be asymptotic to the *y*-axis because at all concentrations below some minimum (threshold) value no measurable adverse response will be elicited, whereas at all concentrations above some maximum value most or all of the exposed test groups will be adversely affected. Figure 15.1 shows the typical sigmoid form of the concentration–response relationship when the probability of a concentration–response is expressed in terms of cumulative response. Each point has variance due to biological variability within a sample of test organisms for each concentration.

For hormetic responses, the shape of the concentration–response may be either an inverted U-shape or a J-shape, depending on the biological response endpoints measured at the low end of the concentration–response curve (Figure 15.2) (Calabrese, 2005). When the response at low exposure concentrations is decreased disease incidence compared to controls, the curve is J-shaped; however, when the response at low exposure concentrations refers to an increase or stimulation in growth or fecundity followed by inhibition at higher concentrations, the curve is an inverted U-shape. For responses at the low end of the curve, there is a reduction in damage and a stimulatory effect 30 to 60% above controls and well below the NOEC; however, as the concentration increases, an exposure concentration will exist where the ability to overcompensate will be negated and net toxicity will result. As Calabrese (2005) pointed out, "The key feature of the hormetic dose response is that it is a normal component of the traditional dose response." In essence, above the no-effect concentrations, the hormetic dose–response and the sigmoidal dose–response are the same.

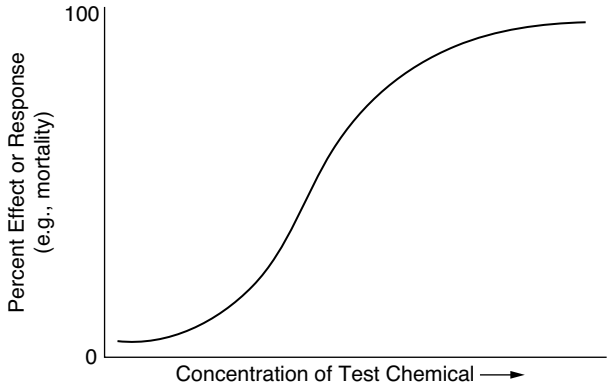

FIGURE 15.1 Typical form of the concentration–response curve.

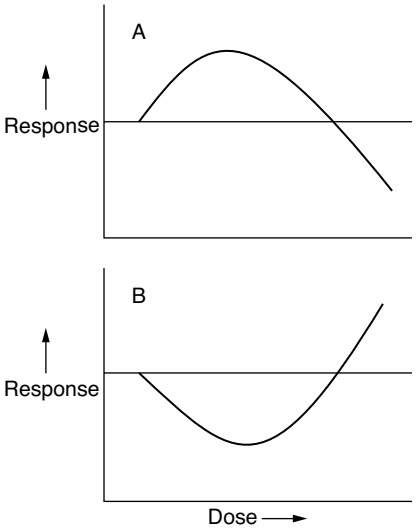

FIGURE 15.2 (A) The most common form of the hormetic dose–response curve depicting low-dose stimulatory and high-dose inhibitory response. Endpoints displaying this curve include growth, fecundity, and longevity. (B) The hormetic dose–response curve depicting low-dose reduction and high-dose enhancement of adverse effects. Endpoints displaying this curve include carcinogenesis, mutagenesis, and disease incidence. (From Calabrese, E.J., *Environ. Pollut.*, 138(3), 379–411, 2005. With permission.)

Criteria for Effects (Endpoints and LC_{50})

Different quantifiable criteria for effects (or measurement endpoints) may be used to express toxicity and to compare chemically exposed organisms with unexposed test organisms. Most often, toxicity test effect measurements are selected based on their biological significance and whether they are readily observable and quantifiable. Effect data may be quantal or graded. Quantal response data are all-or-none types of effects. Graded responses are continuous and quantitative. Mortality and incidences of signs of intoxication are quantal responses, whereas body weight, body length, and neonate production are examples of continuous data. Whether a response is quantal or quantitative, the response is generally expressed as a proportion (e.g., percentage mortalities, percent inhibition). Mortality and survival over a specified period of time are typical effect criteria in short-term (acute) tests. Continuous data are the sublethal effect criteria measured in long-term (chronic) tests. They are measured not in terms of incidence but in units of measured response (e.g., milligrams, centimeters) that can be used to compare chemically

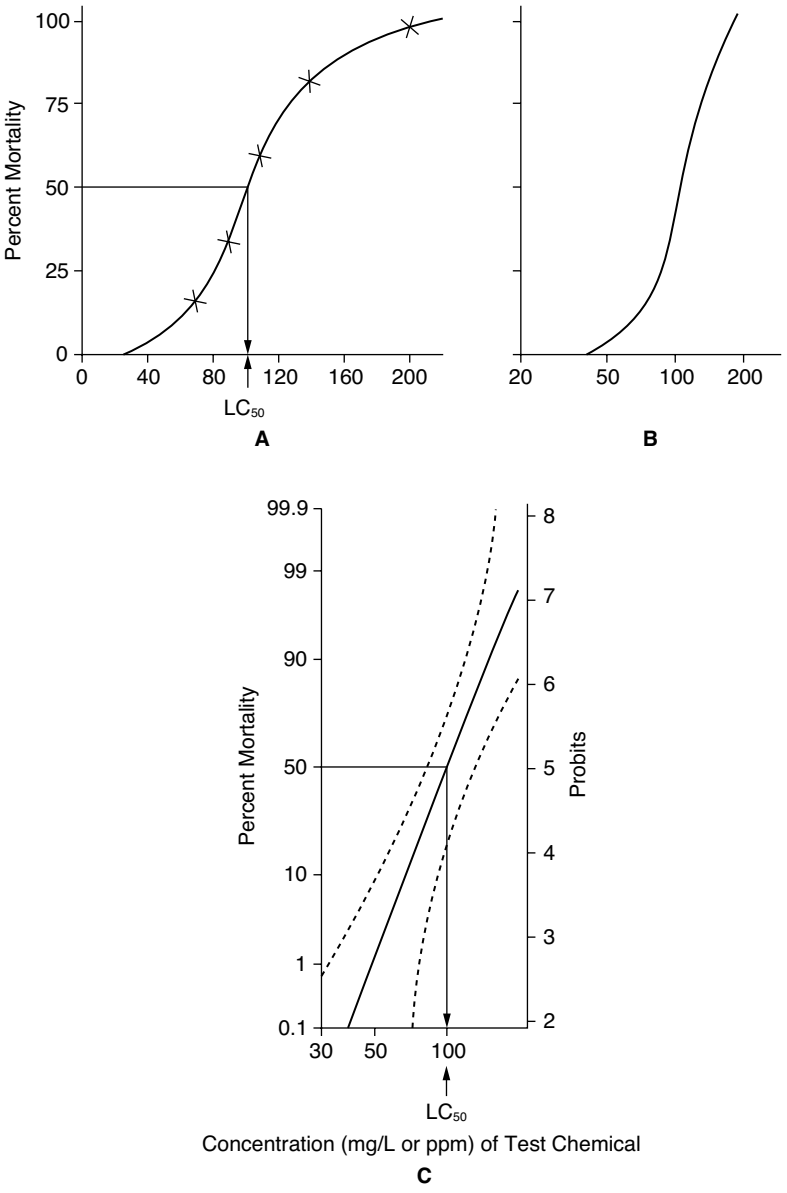

FIGURE 15.3 Mortality in a fish population exposed to a range of concentrations of a chemical in water. (A) Percent mortality vs. concentration plotted on an arithmetic scale. (B) The same data but with mortality on an arithmetic scale and concentration on a logarithmic scale. (C) The same data but with mortality expressed as probits vs. concentration on a logarithmic scale. The dotted lines on each side of the curve represent the 95% confidence limits.

exposed with unexposed control organisms to determine statistical significance. Because there is usually a set sample size of fish for each test concentration (e.g., $n = 10$ fish per concentration), a series of graded measurements is obtained for each concentration. A quantal test determines the presence or absence of response; a quantitative test requires measuring the extent or severity of responses by each organism.

The relationship between the degree of response of test organisms and the quantity of exposure to the chemical almost always assumes a concentration–response form. In Figure 15.3A, the *y*-axis represents percentage mortality (or any effect) and the *x*-axis represents concentration of test chemical; both variables increase with distance from origin. The graph represents results of a test in which groups of test organisms of some species were exposed to graded concentrations of a chemical for a certain time

period. The cumulative responses to test concentrations yield the sigmoid (S-shaped) curve. Each point in the curve represents the average cumulative response to the specific concentration, and each average has the associated variation. The least variability in the curve is at the 50% level of response. The concentration at which 50% of the individual organisms respond (median) after a specified duration of exposure (e.g., 24 or 48 hours) is used as a measure of the activity or toxicity of the chemical. This concentration was adopted as a benchmark because median values are more consistent and tend to have narrower confidence intervals than lower percentiles. Please note that the widescale use of 50% is not as a result of any specific biological significance.

In determining the toxicity of a new chemical to fish, an acute toxicity test is first conducted to estimate the median lethal concentration (LC_{50}) of the chemical in water to which organisms are exposed. The LC_{50}, in simplest terms, is the concentration of a chemical estimated to cause mortality in 50% of a test population over a specified time period. When effects other than mortality are measured, the expression EC_{50} is used. The EC_{50} (or median effective concentration) is the concentration estimated to produce a specific effect (e.g., behavioral, physiological) in 50% of the test population after a specified duration of exposure. The LC_{50} can be interpolated from the curve in Figure 15.3A by drawing a horizontal line from the 50% mortality point on the *y*-axis to the concentration–response curve and then drawing a line from the point of intersection with the curve to the *x*-axis. The vertical line intersects the *x*-axis at the LC_{50} value.

The normally distributed sigmoid curve approaches 0% mortality (or other effect) at low concentrations and approaches 100% mortality as concentration increases. The middle portion of the curve is linear, in regions between 16% and 84% and represents 1 standard deviation of the mean in a normally distributed population. Figure 15.3B is a plot of data in Figure 15.3A, but the logarithm concentration was used to convert to a straight line. Figure 15.3C represents another transformation, with the log concentration plotted against percent mortality expressed as probits. Probit transformation adjusts mortality data to an assumed normal population distribution that results in a straight line.

Probit transformation is derived from the normal equivalent deviate (NED) approach developed by Gaddum (1933), who proposed measuring the probability of responses (i.e., proportion dying) on a transformed scale based in terms of percentage of population or the standard deviations from the mean of the normal curve with a mean = 0 and standard deviation = 1. For example, a NED of 0 would correspond with the median response of 50% (or mortality of 50%). A proportion below the mean by 1 standard deviation or 16% would be a NED of –1. Bliss (1934a,b) suggested that 5 be added to the NED to eliminate negative numbers (when probability has a value of less than 50%). The converted unit of NED plus 5 were called *probits*. The linear relationship between probits and log concentration is similar to the relationship between NED and log concentration. The probit transformation adjusts mortality (proportion dying) or other quantal data to an assumed normal population distribution and yields a straight line.

Confidence Interval (CI)

The degree of scatter of observed values at each concentration may be evaluated and expressed as confidence limits. The limits indicate the range within which the concentration–response line would fall in replicate tests in 19 out of 20 samples (95% CI) taken at random from the same test population under the same set of conditions. A series of such curves will be well correlated with each other at the 50% mortality level but not well correlated as mortality approaches 0 or 100%.

Slope

The cumulative probability of response is directly related to the standard deviates of the log concentration of the population. The slope (response/concentration) of the log concentration–response curve will indicate the relationship between a range of concentrations and the lethal response. The slope may be considered an index of the range of sensitivity to the chemical within the test sample of organisms. The slope is more important in hazard and risk assessment than the numeric value of the LC_{50} because more valuable mechanistic information can be developed. For example, with a steep slope, large increases in mortality are associated with small increases in exposure concentration which indicates faster absorption

and rapid onset of effects. A flat slope, however, reflects small increases in response with large increases in concentration. A large margin of safety is predicted for chemicals with a flat slope. With the slope, it is possible to extrapolate the response to a low concentration (e.g., LC_5, LC_{10}). It is important to report the slope along with the confidence limits. This is especially relevant when comparing chemicals. Chemicals may have identical LC_{50} values but different slopes and thus different toxicological characteristics. Conversely, parallel concentration–response curves may indicate similar mechanism of uptake, mechanism of toxicity, and kinetics. The LC_{50} or slope alone cannot indicate with certainty a mechanism of action, but with pharmacokinetic and biochemical studies it may be possible to determine a mechanism.

The LC_{50} is statistically derived and is not a biological constant. The numeric value of the LC_{50} has been used to classify and compare the toxicity of different chemicals. Although the LC_{50} under a defined set of environmental conditions can provide useful information, the numeric value is not necessarily equivalent to acute toxicity in the field. Lethality is only one endpoint in defining acute toxicity. Defining acute toxicity based only on the numeric value of the LC_{50} is not appropriate; however, it is widely used in the early phases of most risk assessment.

Toxicity Curves

Because mortality (or survival) data are collected for each exposure concentration in a toxicity test at various exposure durations (24, 48, 72, or 96 hours), data can be plotted in other ways; for example, the cumulative percentage mortality (probit transformed) vs. logarithm of time can be plotted for each exposure concentration in a test (Figure 15.4A). The straight line of best fit is then drawn through the points. These are time–mortality lines. The LT_{50} (median lethal survival time) can be estimated for each concentration. The median survival times (LT_{50}) and confidence limits can then be plotted against chemical concentrations to yield toxicity curves. An alternative to these methods is to plot percentage mortality (probit transformed) against chemical concentration (log transformed) for each observation period (24, 48, 72, or 96 hours). Instead of a series of survival–time–mortality curves, the curves are for concentration–mortality (Figure 15.4B). For each observation time, the median lethal concentration (or LC_{50}) can be determined. This numeric value is the concentration producing mortality in 50% of the test organisms within the specified time period. The resulting toxicity curve of LC_{50} against observation time differs only in that confidence limits are expressed in terms of concentration rather than time.

Toxicity curves describe relationships between concentration and survival time in test organisms. For any chemical, there may be a low concentration that will not cause mortality of 50% of test organisms. The curve will become asymptotic to the time axis. The concentration at which this occurs is the threshold median lethal concentration or incipient lethal level. The incipient LC_{50} is the concentration at which 50% of the test population can live for an indefinite time, or the lethal concentration for 50% of test organisms in long-term exposure. Furthermore, even at very high chemical concentrations, death may not be immediate and may take time to occur. The curve will be asymptotic to the concentration axis. Between the two asymptotes, the curve will show a decrease in survival time as concentration increases. The most important characteristic of a toxicity curve is that it indicates the threshold median lethal concentration. Toxicity tests should be conducted until a lethal threshold is determined. Results of acute tests must be carefully interpreted unless toxicity curves and thresholds are obtained. It should be noted that many acute toxicity tests are not continued long enough; therefore, acute toxicity tests for slow-acting chemicals may underestimate toxicity.

Criteria and Approaches

Fish toxicity tests are used to evaluate the concentrations of a chemical and duration of exposure required to produce the criterion effects. Most toxicity tests use protocols with standardized methodologies with designs that are based on concentration–response relationships and the basic toxicology principles discussed earlier. The criteria used to determine the suitability of a standard test include the following:

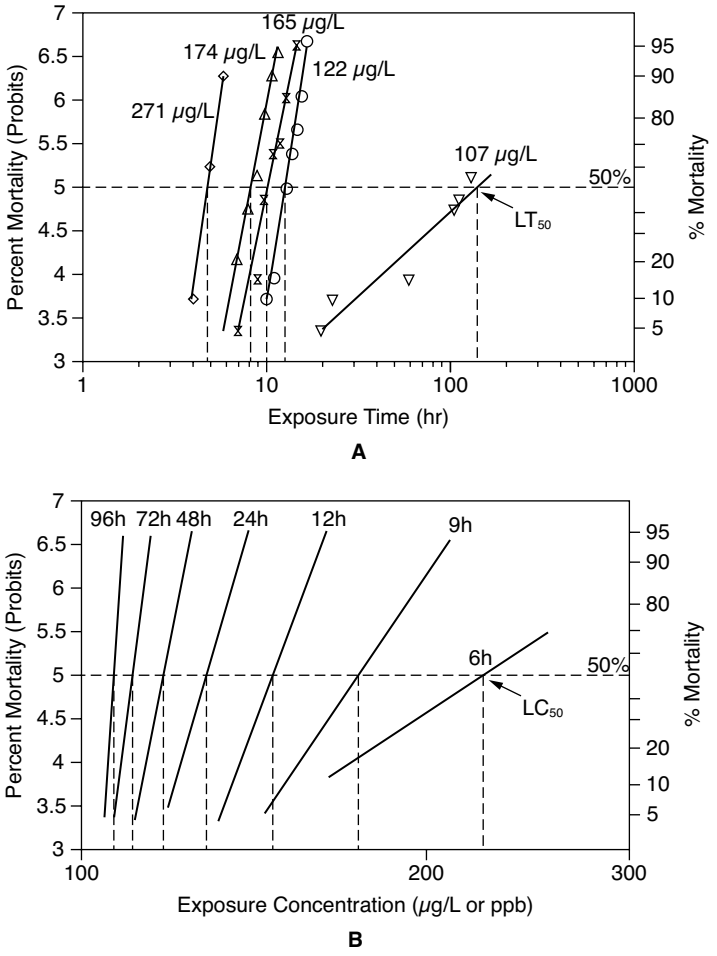

FIGURE 15.4 Cumulative percentage mortality for a fish population exposed to a range of concentrations of a chemical in water. (A) Plot of exposure time vs. mortality at various exposure concentrations (drawn from data for low-fat fish exposed to pentachlorophenol). (B) Plot of exposure concentration vs. mortality at various exposure times (idealized pentachlorophenol results). (From van den Heuvel, M.R. et al., *Aquat. Toxicol.*, 20, 235–252, 1991. With permission.)

- The test should be widely accepted.
- The test should have a sound statistical basis and should be repeatable in different laboratories; thus, tests must be standardized and conducted according to defined protocols.
- Data should include the results of effects from a range of concentrations with realistic exposure times.
- Laboratory tests should have predictive potential for defining field effects and should be useful for hazard and risk assessment.
- Test procedures should be easy to carry out.
- Tests should be sensitive to be able to detect the effects under investigation.

Test methods in the laboratory are designed to examine the responses of a few individuals within a species to chemical exposures to establish cause-and-effect relationships. These tests can also provide data on the relationship between external and internal concentrations of a chemical, the duration of exposure, and the biological effects. Single-species tests are straightforward and standardized and can be replicated. Single species tests are currently conducted with species representative of broad classes

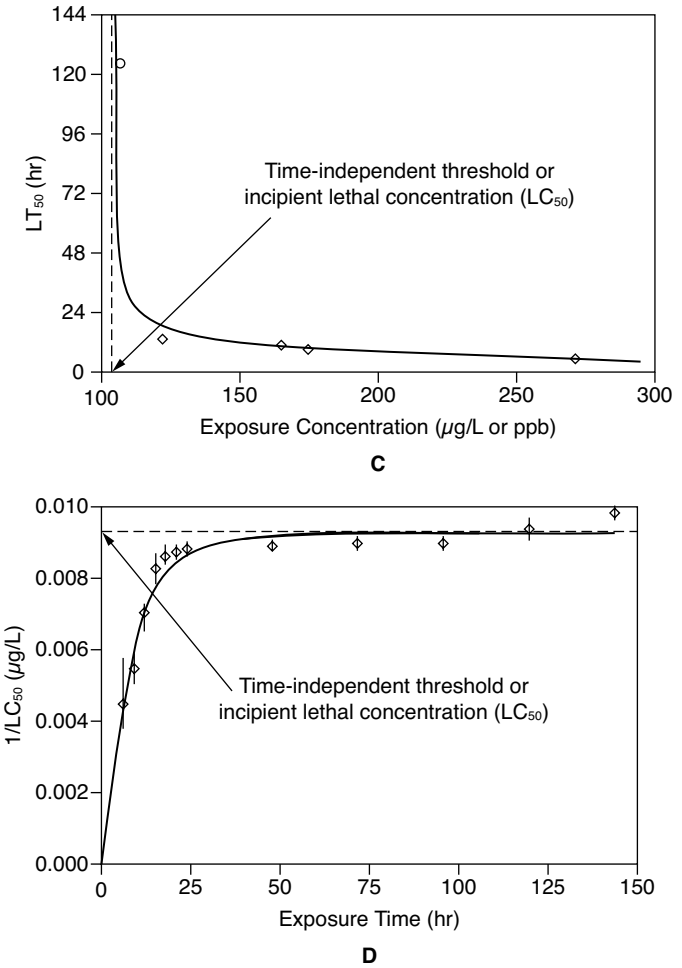

FIGURE 15.4 (cont.) (C) Plot of LT_{50} (median time to death) vs. exposure concentration (LT_{50} values from part A). (D) Plot of the inverse of LC_{50} vs. exposure time (LC_{50} values for low-fat fish exposed to pentachlorphenol). (From van den Heuvel, M.R. et al., *Aquat. Toxicol.*, 20, 235–252, 1991. With permission.)

of organisms (fish, invertebrates, algae). The utility of single-species fish tests is a function of the criteria used to select test organisms (see below). The main limitation is that effects in the laboratory may not occur at all or to the same degree at similar concentrations in the environment. Effects measured in the laboratory are a result of the inherent variability of the test fish population under constant conditions. Furthermore, bioavailability of chemicals is optimized because filtered laboratory waters are used instead of natural waters. These tests do not consider species interactions and environmental influences. Because most tests are conducted with water exposures, sediment and food exposures are rarely considered. The latter exposure routes become particularly relevant with chemicals that are hydrophobic.

Small simulated model microcosm tests can also be conducted in the laboratory using a multispecies approach. Laboratory microcosms are small-scale enclosures (e.g., fiberglass, glass, plastic) containing a multitude of samples (e.g., water, sediment, one or more species of fish, invertebrates, and plants). Any combination or all of the latter samples may be incorporated into the test depending on study objectives. The advantage of this approach is that effects beyond the level of direct effects on a single species can be studied, including indirect effects. Results provide information more predictive of the ecological consequences of chemical release; however, environmental conditions are still controlled in laboratory microcosms but replication may be easily obtained. Although cause-and-effect relationships

are also more easily analyzed than in natural systems, there are several limitations. Temperature and seasonal fluctuations and certain biotic components (invaders) not represented can significantly impact results. Furthermore, all fish may not be adequately represented. Currently, no standardized laboratory microcosm protocols with fish are available; however, standardized protocols are available for first (primary consumers) and secondary (grazers, herbivores) trophic-level organisms including the detrital (or recycling) level (ASTM, 2000).

Ideally, data from laboratory and field studies should be obtained for certain chemicals before widescale use. In designing studies, considerations should be given to:

- *Purpose (or objective) of the test*—Tests used to develop numerical water or sediment quality criteria for chemicals may be different than those tests used in support of chemical registration or for routine monitoring or screening of effluents. Testing for a pesticide to be used on a specific crop or an ornamental, for example, may not require as extensive a test battery as that required for the development of numerical criteria.
- *Characteristics of chemical*—Chemicals with low solubility, volatility, susceptibility to sorption or degradation, or other factors that affect their behavior will require special steps so the results of toxicity tests are indicative and relevant to the way the chemical will behave in the environment. Chemicals that degrade rapidly and that are not analytically detectable in natural waters after a few days may not require tests with long-term exposures. Measurements of test concentrations should be a requirement in all studies.
- *Mode of action of chemical*—Knowledge of the mode of action of the chemical will provide information on the most likely biological endpoints to measure during and at the end of toxicity tests.
- *Pattern of use of chemical (or discharge)*—A test that uses constant and continuous chemical exposure will produce inaccurate results if organisms are only intermittently exposed. The latter is important when considering that most pesticides in the field are used at different rates with various time intervals in between applications, although chemical safety testing for pesticide registration requires continuous exposure to constant concentrations of a pesticide.
- *Interactive effects*—Laboratory testing of single chemicals should consider any factors that may affect toxicity and may include characteristics of the water (e.g., dissolved oxygen, humic substances), temperature changes, and exposures to more than one chemical.
- *Impact on characteristics of the ecosystem*—Natural waters representative of oligotrophic, mesotrophic, and eutrophic freshwater systems should be considered in toxicity tests. Whenever possible, natural saltwater should also be used in testing instead of prepared saltwaters.
- *Characteristics of test organisms*—Indigenous non-target organisms should be considered in testing, whenever possible.

No single test can provide enough information to assess the impact of toxicants on the aquatic environment. Ideally, a combination of tests should be used in the laboratory and field.

General Test Design

Fish toxicity testing in the laboratory follows a tier approach, progressing from simple short-term tests to more complex longer-term tests based on results of previous tests. Although specific details of the protocol for each toxicity test may differ, certain basic features are fundamental to the design and analysis of all toxicity tests. Each protocol requires careful control and monitoring of conditions such as pH, temperature, dissolved oxygen, exposure concentration, photoperiod, and water chemistry. Fish are exposed in test chambers (e.g., glass tanks) to three to five different concentrations of a test material (e.g., pesticide, industrial effluent, industrial chemical) in dilution water solutions. The criteria for effects (e.g., mortality, growth, reproduction) and the type of test (i.e., acute or chronic) are established before testing and are then evaluated by comparing chemically exposed (treated) organisms with

untreated organisms (controls). All toxicity tests must include an untreated control to ensure that the observed effects are associated or attributable to exposure to the test substance. The three types of controls are:

- The classical untreated (negative) water control consists of the same conditions as the treated groups except for chemical exposure. Effects related to the health of the organisms and quality of dilution water may be ascertained from this control.
- The solvent (or vehicle) control is used in tests with poorly soluble test materials. The solvent is used as a carrier to make the test material more miscible with water. The solvent control is untreated except that the maximum volume used to prepare a test material concentration is added. This control takes into account effects of the solvent alone on test fish. The toxicity of the solvent should be relatively low compared to the test material. Solvents used in fish toxicity include acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), and triethylene glycol (TEG).
- Positive (or reference) control is a material known to produce a defined effect in fish (or in other organisms). An ideal reference toxicant is toxic at low concentrations, produces the desired effect (endpoint) rapidly, and is stable, nonselective, and detectable by analytical techniques. It is used to determine the health and sensitivity of organisms, to compare relative toxicities of substances by using the control as an internal standard, to perform interlaboratory calibration, and to evaluate reproducibility of test data. Compounds used as reference toxicants include sodium pentachlorophenate, dehydroabietic acid, phenol sodium azide, p,p'-DDT, dodecyl sodium sulfate, antimycin, and sodium chloride. Environment Canada (1990) compared four organics and nine inorganics as reference toxicants. The most suitable reference toxicants were two organics (sodium pentachlorophenate and phenol), and two inorganics (hexavalent chromium and zinc).

Although thus far little has been done to verify that reference toxicants reflect poor health of organisms or genetically different stocks, they are useful in intra- and interlaboratory quality assurance and quality control. Chemical analyses should be conducted to determine the concentrations to which organisms are exposed (i.e., in water or food). Chemical residues should also be analyzed in tissues of organisms. All analytical chemistry methods must be validated prior to test initiation.

Test Organisms

Several criteria should be used in selecting fish species for testing:

- Widely available fish species with adequate background information should be used.
- Fish species with a known history of diverse sensitivities and behavioral (i.e., reproductive, feeding) strategies should be used.
- Indigenous, representative fish species of the receiving system being studied should be considered first. If definable and possible, a *keystone* fish species should be used. Keystone species are those that influence a community because they play a significant role in the community, not because of their numerical dominance.
- Species must be amenable to culturing and maintenance in the laboratory.

No representative, standardized fish species exists that can be used to answer all questions and evaluate impacts to all ecosystems. Because of variation in responses between fish species to the same chemical (i.e., both for adults and early life stages), it is necessary to test several different fish species from both fresh- and saltwater systems. In addition, when sediment-bound chemicals are a significant exposure route, different species of bottom-dwelling fish should be considered for testing along with fish that have feeding habits that bring them in intimate contact with sediment during parts or all of their life

cycle. Fathead minnows, bluegill sunfish, largemouth bass, rainbow and brook trout, channel catfish, sheepshead minnows, silversides, and killifish have been widely used in toxicity testing with fish. Test species can be collected from wild populations, cultured in the laboratory or purchased from a commercial supplier; however, all fish species in a test should be from the same source.

Water Exposure Systems

In aquatic toxicity tests, the control and treated fish can be exposed to untreated dilution water or test-water solutions containing the chemical (i.e., effluent) by four techniques:

- In a *static system*, organisms are exposed in still water. A test substance is mixed with water to desired concentrations. Fish are then placed in test chambers and monitored for the entire test.
- In a *recirculation test*, test solutions and control water run through a filter to maintain water quality but not reduce the concentration of the test chemical. Water is returned to the test chamber. This test is similar to static tests but is more expensive, and there is much uncertainty about the effect of filtering.
- In a *renewal test*, solutions and control water are renewed periodically by transferring fish to chamber with freshly prepared test chemical in water (or water alone for controls).
- In a *flow-through test*, test solutions and control water flow into and out of test chambers. The once-through flow may be intermittent or continuous. A stock solution of test chemical is mixed with dilution water, and metering pumps or diluters control flow and volume of stock solutions and dilution water so proper proportions of each are mixed and delivered to test chambers.

Static and flow-through systems are the most widely used means of exposing fish in laboratory acute and chronic toxicity tests. Exposure concentrations are measured during static and flow-through tests, but occasionally calculated numerical values are used in static tests. Static tests are inexpensive to conduct and may be used as an initial screening tool, but they do have disadvantages:

- The test material concentration in the water may not be consistent throughout the duration of a test that is typically 24 to 96 hours.
- Test material may have a high biological oxygen demand (BOD), so the chemical toxicity is masked by dissolved oxygen depletion.
- Metabolic products from organisms may build up in the tanks, producing toxic effects.

Static tests may not accurately represent the effects of the test material under investigation, so exposures beyond 24 hours should make use of flow-through techniques. Flow-through techniques result in more uniform test conditions where test concentration, dissolved oxygen, and other water quality parameters remain constant while waste products are removed. Both techniques are used for acute tests, but for subchronic and chronic tests the flow-through technique is preferred because of the greater consistency in controlling conditions during the test period. As a result, cause-and-effect relationships can be more accurately established.

Standard Procedures

A variety of toxicity test methods using fish have been developed and standardized by different groups, including the U.S. Environmental Protection Agency, Environment Canada, the Organization for Economic Cooperation and Development, and the American Society for Testing and Materials. See Adams (1995), Cooney (1995), Middaugh et al. (1993), Solbe (1993), and Ward (1995), for more information on the types of standard laboratory toxicity tests with fish. Standardization facilitates the comparison and replication of data, allows laboratories with different personnel to more easily conduct a test, offers

ease of use in environmental litigation, and is useful for routine monitoring. Standardization can be achieved by:

- Use of standard, well-written, and validated protocols
- Use of standard test species and disease-free organisms
- Use of reference toxicants

Standard test procedures, however, may not always be applicable for answering certain questions about a particular aquatic system; for example, when evaluating the hazard of a chemical (or effluent) to an indigenous fish community in a specific fresh- or saltwater system, it may not be practical to use standard test species that are not normally present in these bodies of water. Furthermore, it may be desirable to use water characteristic of the system and not standard laboratory-filtered water. Toxicity test protocols also specify that exposures of organisms are for fixed and continuous concentrations of test substances for a defined time period. As pointed out earlier, chemicals rarely enter the aquatic environment at a continuous, fixed concentration but rather intermittently as pulses (or "slugs") or as a one-time spill; therefore, realistic environmental exposures typically include a one-time high exposure concentration usually diluted to lower concentrations with time. A typical standard test in which all test conditions are kept constant or maintained optimally may be inappropriate for predicting responses in a changing natural system. In this case, site-specific conditions should be considered. This type of test design should be customized to the situation, and a standardized test design would not be applicable. Alternatively, if results are to be used to compare the toxicity of one chemical to another, rigid standardization is necessary.

Description of Test Methods

As previously pointed out, toxicity test methods may be categorized according to the length of exposure, test situation, criteria for effects to be evaluated, and organisms to be tested. Some of the commonly used tests and endpoints (effect criteria) that they measure are described below.

Acute Toxicity Tests

Before a definitive acute test is carried out with a particular chemical and species, few to no data may be available; therefore, a range-finding acute test is conducted to pinpoint exposure concentrations for the definitive acute test. Range-finding concentrations tested are normally spaced at intervals of a factor of 10, and only two to four fish may be used per concentration. When the range-finding test defines the smallest range between concentrations that produce mortality and no effects, the definitive test is conducted with at least three, preferably five, concentrations to define the endpoint with greater accuracy and precision. Accuracy is attained by closer spacing between concentrations and precision by using more fish per concentration. Common acute effect criteria for fish include mortality; however, abnormal behavior observations are also noted (e.g., swimming behavior, loss of equilibrium, color changes).

Tests are conducted for a predetermined length of exposure to estimate the 24-, 48-, 72-, or 96-hour LC_{50}; 95% confidence limits; and slope of the concentration–response curve. The LOEC and NOEC have also been reported in acute tests, but the LOEC and NOEC have shortcomings in that they are affected by the study design (e.g., concentrations chosen, number and spacing of replicates in each concentration) (Chapman et al., 1998). Much uncertainty is associated with these values, as they are statistically determined using hypothesis testing, they do not have confidence intervals like point estimates (e.g., LC_{50}, EC_{50}), and they can only be compared by their ranges of values. In assessing acute hazards, the aquatic toxicology community is moving toward benchmark values such as the confidence interval that result in an LC_{10} as a no-effect concentration. An acute test may also have a duration that is not predetermined, in which case it is referred to as a time-independent (TI) test. The acute TI test should continue until acute toxicity (e.g., mortality) has stopped or nearly stopped and the toxicity curve indicates that a threshold (or incipient) effect concentration can be estimated.

TABLE 15.3

Endpoints Measured in Subchronic and Chronic Exposure Tests with Fish at Different Stages

F_0 embryo
Incubation time from fertilization to hatch
Hatching time required for embryos to hatch
Time from complete hatch to first feeding
Percentage of normal and abnormal larvae at complete hatch
Weight of larvae at complete hatch
F_0 larvae and juveniles
Abnormal behavior
Total percentage of deformed fish
Percent survival
Weights/lengths of juveniles
Number of juveniles that develop to sexual maturation and develop secondary sex characteristics
F_1 embryos
Number of eggs produced by sexually mature fish
Number of viable embryos (F_1); hatching, survival, and growth of F_1

The specific environmental conditions and measurements in acute fish tests are delineated in the standardized tests. Early work with acute toxicity tests relied on reporting nominal concentrations of chemicals (based on stock solution calculations), rather than concentrations based on analytical measurements. All acute definitive tests should be based on measured concentrations. When possible, chemical residues in fish tissue should also be measured. This will provide data to equate lethal body residues with acute toxicity endpoints (e.g., LC_{50}, LC_{10}) under optimum conditions of bioavailability. It will further enable one to evaluate the meaning of realistic environmental exposure concentrations (EECs) of chemicals.

In analyzing acute toxicity data, the time to death (i.e., survival time) or duration of exposure before death should also be obtained (Sprague, 1969) to determine the probability of dying during a given interval. This is especially relevant for acute exposures that typically occur in the environment. This endpoint will become more applicable when acute exposures in the laboratory implement procedures that incorporate different natural waters (e.g., oligotrophic, mesotrophic, and eutrophic) and sediment into acute testing procedures. To date, acute toxicity tests with fish have used laboratory-filtered waters without sediment.

Chronic Toxicity Tests

Objectives of subchronic and chronic exposure tests are listed in Table 15.3. As pointed out earlier, in full life-cycle chronic tests the test organisms are exposed for an entire reproductive life cycle (egg to egg) to at least five concentrations of test substance. Partial life-cycle tests involve several sensitive life stages and include reproduction and growth during the first year but do not include early juvenile stages. In full chronic tests, exposure may start with the egg or zygote and continue through development and hatching of the embryo, growth and development of the young organism, attainment of sexual maturity, and reproduction to produce a second-generation organism. Tests may also begin with the exposed adult and continue through egg production, fry, juvenile, and adult to egg. Effect criteria include growth at different stages, reproduction, development of gametes, maturation, spawning success, hatching success, survival of larvae or fry, growth and survival of different life stages, and behavior. Specific endpoints measured in subchronic and chronic tests are listed in Table 15.3.

Hypothesis testing is used to detect statistical differences in effect criteria data generated from chronic tests. Data are analyzed to determine whether responses at different exposure concentrations are different from control (or solvent) responses. The experimental design most often used in hypothesis testing for chronic data is a one-way analysis of variance (ANOVA). A series of treatment concentrations are

randomly assigned, including a control (and solvent or reference, if applicable) treatment, with replications at each treatment level. Observations are made for each treatment for each effect criterion and are assumed to be independent. The assumptions of ANOVA (i.e., equal variances among treatments and normally distributed data) must be met. Transformations of quantal data, proportions (e.g., percentage larvae deformed), or other response expressions are sometimes necessary to meet the requirements of homogeneity of variance and normality. If the ANOVA results lead to rejection of the null hypothesis (H_0 = no difference between treatment means), the treatment (i.e., exposure concentration) had a significant influence on mean response (i.e., biological endpoints measured), but we do not know which means were significantly different. A series of post-ANOVA methods can be used to identify which treatment concentrations differ from each other. When data or transformations of data violate assumptions of ANOVA, statistical power may be sacrificed by using nonparametric, post-ANOVA tests. For a complete review of statistical procedures used for analyses of chronic, sublethal effects data, see Newman (1995).

At the end of a chronic test, the LOEC and NOEC are determined for each endpoint measured. In addition, the maximum acceptable toxicant concentration (MATC) is estimated for the endpoint with the lowest NOEC and LOEC. The MATC is the threshold concentration of a chemical within a range bounded by the NOEC and LOEC. For regulatory purposes, it is calculated as the geometric mean of the LOEC and NOEC. The MATC has no statistical confidence interval because the LOEC and NOEC are used to define it. The MATC should not be extrapolated to predict a safe concentration because it is a reflection of test design, species, and exposure duration; however, a range of MATCs from chronic tests with different fish species will provide more supportive data to extrapolate to biologically safe concentrations, especially when natural waters are used for chronic exposures.

The MATC can also be used to generate application factors (AFs) for chemicals. The AF is derived as the numerical value of the ratio of the MATC to the LC_{50}. The assumption is made that the AF for a given chemical is constant over a range of test species; therefore, if an AF is derived for one species with actual MATC and LC_{50} data, then the MATC could be derived for a second species. The AF for the first species and the LC_{50} for the second species could be used to estimate the MATC for the second species. The use of arbitrary application factors with LC_{50} values to predict chronic toxicity and safe concentrations must be approached with care. The acute-to-chronic toxicity ratio (ACR) is a variant of the AF and is the inverse of the AF and is also used to estimate an MATC for species when only acute toxicity data are available.

Short-Term Sublethal Effects

In the development of test procedures to evaluate the toxicity of whole effluents (e.g., municipal or industrial wastewaters) to aquatic organisms, the U.S. EPA developed short-term sublethal tests (i.e., 7 to 9 days or less; often misleadingly called short-term chronic tests) that focus on the most sensitive life-cycle stages. See Dorn and van Compernolle (1995) for a summary of short-term toxicity tests for whole effluents with fish. Fish are exposed to five different effluent dilutions (e.g., 100, 50, 25, 12.5, and 6.25%), including untreated controls, with replication to determine magnitude of toxicity; however, in compliance monitoring (i.e., in discharge permits) an option is to choose five concentrations that bracket the receiving water concentration (above and below). This monitoring would determine compliance status (i.e., meets or exceeds permit requirements) as well as estimate the NOEC.

In short-term sublethal tests, the LC_{50}, EC_{50}, NOEC, and LOEC are reported based on percent effluent to which organisms are exposed. Effects may include changes in growth, survival, or percent hatch, for example. To overcome some of the problems in statistically deriving the NOEC and LOEC, the inhibition concentration (IC) may be used which is a point estimate interpolated from the effluent concentrations at which measured effects occurred in the sublethal test. The IC is an estimate of the effluent concentration (i.e., percent effluent) that would cause a given percent reduction in a biological endpoint of the test organism. An IC_{10} for growth, for example, would represent the percent effluent at which a 10% reduction in growth occurred. This approach is similar to determining the LC_{10} or EC_{10} when organisms are exposed to a single chemical. Because the IC is a point estimate, a confidence interval can be calculated.

If an effluent is found to be toxic at concentrations exceeding the allowable instream dilution or other permit stipulation, the discharger is required to conduct a toxicity identification evaluation (TIE) and toxicity reduction evaluation (TRE) (Dorn and van Compernolle, 1995; U.S. EPA, 1991, 1992, 1993a,b, 1996). The TIE is a three-phase procedure where the effluent is manipulated to measure changes in toxicity. The TIE, in general, is performed to try to identify the fraction or procedure that has been effective in removing toxicity or affecting toxicity changes. When the fraction has been identified, chemical identification is initiated and subsequent spiking or back addition of the identified chemical is performed for confirmation. The TIE is a single-sample analysis and should be repeated several times and confirmed for several species. Based on results of the TIE, a decision is made whether to conduct the TRE using source identification (i.e., which will lead to control methods) or to conduct treatability studies on the final effluent itself (which will lead to identification of possible treatment methods for the effluent). A TRE focuses attention in toxicity reduction procedures that will enable a permittee (e.g., industrial company, municipality) to come into compliance with water quality standards.

Bioaccumulation Tests

Bioconcentration tests are designed to determine the bioconcentration factor (BCF). The BCF is the ratio of the average concentration of test chemical accumulated in tissue of the test organisms under steady-state conditions to the average measured concentration in water to which organisms are exposed and is unitless. Bioaccumulation is a general term describing the net uptake of foreign chemicals from the environment by any or all routes (respiration, diet, dermal) from any source in the aquatic environment (i.e., water, dissolved, colloidal, particulate organic carbon, sediment, other organisms). Bioconcentration is a more specific term reserved for describing accumulation of chemicals from water alone.

Bioaccumulation is of concern for its potential effects on the organism and for contamination of higher trophic levels, including humans. It should be noted, however, that the presence or accumulation of chemical residues in organisms is not an adverse effect by itself; only the biological responses produced by such residues have potential adverse effects.

Chemicals with molecular weights between 100 and 600, with low water solubility (ppb, low ppm), high log K_{ow} (>2), and high K_{oc} (>500) have typically been considered for bioconcentration testing. In addition, chemicals with a high proportion of halogenated aliphatic or aromatic substituents or highly branched aliphatic moieties have the highest persistence and are most likely to bioaccumulate.

Standardized bioconcentration tests with fish have typically been conducted with single-chemical (e.g., non-ionizable organic chemicals, metals) exposures in water. Fish are exposed to a constant, continuous sublethal concentration (e.g., 96-hr LC_{50}/acute-to-chronic ratio) of the chemical in water. If a concentration in natural water is available, it will be used as the test concentration. Exposure continues until an apparent steady state is reached (i.e., the BCF that does not change over 2 to 4 days or the BCF when uptake and depuration are equal). The bioconcentration test consists of an uptake phase (approximately 3 to 4 weeks) followed by a depuration phase (2 to 3 weeks). During the uptake phase, a flow-through system is used. The flow of water should be sufficient such that the organisms do not significantly deplete either the chemical or the oxygen in their surroundings. The test water should be analyzed before and during the uptake phase (e.g., once every three days) to verify exposure concentrations. An untreated control should be maintained throughout the experiment to provide uncontaminated samples. Organisms are sampled for tissue residues (e.g., once every 3 days) during the uptake phase to establish the plateau or steady-state concentration.

After the uptake phase, the depuration phase begins in which organisms are transferred to uncontaminated water, and this phase is usually continued until the concentration in organisms is less than 10% of the steady-state concentration or is below the detection limit in tissue. Water and tissue sampling times are similar to those for the uptake phase. Fish are fed daily during both phases of the test. Duplicate water samples and triplicate residue analyses of the organisms are taken at the end of each sampling time in the uptake and depuration phases. Residues are reported on a whole-body, wet-weight basis; however, for special studies, residues in separate tissues (e.g., muscle, fat, blood, fillet) or concentrations expressed on the basis of dry weight or lipid weight may be desirable.

When apparent steady state is reached, the BCF should be calculated as the geometric mean of BCFs obtained during steady state, along with calculation of the 95% CI. If apparent steady state is not reached, the BCF at the end of the uptake phase should be calculated. The uptake rate constant, depuration rate constant, and projected steady-state BCFs and 95% CIs should be calculated using a model.

The BCFs and rate and extent of uptake and depuration depend on water quality, species (age and size), physiological conditions, and other conditions. Furthermore, natural systems contain particulate and colloidal matter not present in laboratory systems. Chemicals with low water solubilities will substantially sorb to these types of matter in natural systems. Sorption will decrease the bioavailability for some species but may increase the bioconcentration for other species that ingest particulate matter; food may be an important source of chemical residues for certain fish. Bioconcentration tests with chemicals should therefore consider other routes of chemical exposure; for example, test chemicals can be incorporated into sediment or food or mixed with fine sediment particles. Fish with different behavioral strategies should be used in bioconcentration tests.

Results of bioconcentration tests are important in assessing hazard and risk and in deriving sediment and water quality. When designing an aquatic toxicology program, the type and extent of bioconcentration and bioaccumulation testing depend on the characteristics and fate of the chemical, as well as the types of exposure, target systems, and organisms affected.

Toxicity Testing: Summary

Toxicity testing with fish in the laboratory has many advantages. A well-designed testing program using a variety of indigenous fish species with different behavioral strategies, natural water (including sediment), and realistic chemical exposures can be useful in hazard and risk management decisions. In spite of the uncertainties and the fact that tests cannot be conducted under all possible exposure scenarios, more realism can be incorporated into all toxicity regulatory requirements. In addition, because significant biological activity may occur below the traditional NOECs, traditional regulatory tests should therefore incorporate additional exposure concentrations at the low end of the concentration–response curve to define this critical area. The public can easily relate to fish because of sport fishing and their economic importance; therefore, when adverse effects on fish are observed, the public takes notice.

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16

Biomarkers

Daniel Schlenk, Richard Handy, Scott Steinert, Michael H. Depledge, and William Benson

CONTENTS

Introductory and Historical Perspectives

Understanding basic mechanisms of toxicological processes is integral to assessment of risk. In aquatic toxicology, however, the mechanistic evaluation of environmental chemicals is a much younger area of investigation than in mammalian systems and only recently has received significant attention. Prior to the 1970s and 1980s, the development of standardized bioassays and routine environmental monitoring served as the primary means to assess and regulate chemical contaminants. Subsequent to this era, investigators in aquatic toxicology turned their attention from whole organism responses to those at the cellular or organ system levels of biological organization. This led to a proliferation of efforts and information relating to clinical approaches utilizing hematology, histology, histochemistry, metabolism, pharmacokinetics, and physiological or biochemical effects as measures of toxicity. In the l990s, these approaches broadened with the increased use of molecular biology techniques that have greatly assisted efforts to define and understand mechanisms of action. Since 2000, with the recent advances in -omic methodologies, multiple endpoints have been identified as being modified by exposure to various stressors with the potential development for stressor-specific responses. The results of mechanistic studies have proven to be valuable to many applied areas of aquatic toxicology. In risk assessment, mechanistic data have been useful in demonstrating that an adverse effect observed in the laboratory is directly related to population-level effects. As costs associated with environmental compliance increase, regulators and the regulated community, must make the most effective use of the funds and time allocated for reducing environmental impacts. At present, assessment and regulatory decisions continue to be based primarily on empirical measures that may be highly susceptible to change. Decisions based on a more complete mechanistic understanding of chemical effects would be less likely to vary with technological trends. Additionally, the more sensitive our assessment methodologies become, the earlier adverse effects of environmental chemicals on aquatic ecosystems can be measured and, in turn, the more accurate the evaluation of ecological risk.

Adverse effects to aquatic organisms begin with the release of a chemical into the environment. A much relied upon means to evaluate ecological risk has been through environmental monitoring in which chemical residues are assessed. This approach has provided useful information but with significant limitations, not the least of which are the time and costs associated with chemical residue analysis. Although time and cost restraints are arguable issues, a more significant challenge associated with such an approach is the inability to quantitatively evaluate the availability of a chemical from the environmental matrix to the aquatic organism. Furthermore, metabolism or limitations in available technology may render a chemical difficult, if not impossible, to detect in environmental or biological samples. Application of biomarkers in environmental monitoring may resolve many of these challenges by providing a measure of availability of an environmental chemical to an aquatic organism by providing a direct measure of the response of an organism to chemical exposure. Regarding biological response to sublethal concentrations of environmental chemicals, Depledge et al. (1993) noted that an essential criterion of the biomarker approach is the identification of early onset changes in otherwise healthy organisms that predict increased risk of development of chemically induced pathologies. Other potential uses are listed in Table 16.1.

TABLE 16.1

Potential Uses for Biomarkers in Field Studies.

To demonstrate residency or exposure for the purposes of interpreting responses as site specific
To demonstrate or define the characteristics of an unknown chemical or chemical mixture
To demonstrate bioavailability (or lack thereof)
To examine the time course of uptake
To provide *in vitro* opportunities for understanding mechanisms
To prioritize sites, stressors, or samples for further sampling or analyses
To direct testing during fractionation procedures to isolate unknowns
To evaluate the time course or success of remediation
To conduct surveillance

TABLE 16.2

Comparisons of Risk Assessment Components and Biomarker Types

Risk Assessment Components	Biomarker Type
Exposure assessment	Exposure
Effects assessment	Effect
Uncertainty analysis	Susceptibility

During the past two decades, attempts have been made to identify and characterize biomarkers in a range of organisms from bacteria to humans to predict disease or detrimental ecological effects (Adams, 1990, 2002; Decaprio, 1997; Depledge et al., 1993; McCarthy and Shugart 1990; Shugart et al., 1992). Because this text is focused specifically on piscine species, discussions are limited to relationships in fishes. The term *biomarker* represents many endpoints, and several groups have challenged its original definition. Several definitions of biomarkers have been proposed since the first consensus definition proposed by the Committee on Biological Markers of the National Research Council (NRC) (1987). The NRC defined biomarkers as "indicators signaling events in biological systems or samples following chemical exposure" and proposed the use of biological markers to determine: (1) internal dose or biologically active concentration (exposure), (2) adverse effects, and (3) susceptible populations or individuals in an attempt to predict and possibly prevent clinical disease, specifically in humans. In fact, in the original definition and classification by the NRC (1987), the emphasis was placed on human health, specifically associated with reproductive toxicity. With fish specifically in mind, Adams (1990) modified the original NRC definition to include characteristics of organisms, populations, or communities that respond in measurable ways to changes in the environment. As the measurements have proceeded to include other organisms such as fish, debate has occurred as to their utility as a "marker" or as an "indicator" in ecological settings (McCarty and Munkittrick, 1996). It has been further argued that studies examining a biological response without a definitive purpose are essentially useless as "indicators" (Holdway, 1996). Peakall (1992) suggested the term *biomarker* to indicate effects relating to individual organisms and *bioindicator* to indicate effects measured at the population or community levels of biological hierarchy. It is clear from the multiple definitions of the term *biomarker* that any study using this terminology must begin by defining the specific aims and purposes of the biological response that is measured or proposed as a biomarker.

The NRC proposed three types of biomarkers in an attempt to classify responses as markers of exposure, effect, and susceptibility. Each of these definitions has been addressed previously and discussed in terms of its potential use in ecological risk assessment paradigms (Schlenk, 1999). As more biomarkers have been increasingly proposed and characterized, significant overlap may occur when using this nomenclature, as some biomarkers can be in each of the three capacities (Table 16.2). An effect resulting from stressor exposure may be defined as an early adaptive nonpathogenic event or as a more serious altered functional event, depending on the toxicokinetics and mechanism of action of the compound (Decaprio, 1997). Likewise, biomarkers of exposure and effect may often be combined into a single classification, with susceptibility occurring along any stage (Barrett et al., 1997). For the purposes of this chapter, the three main categories are still subdivided for ease of discussion; however, it should be noted that many markers may be used in one, two, or all three categories simultaneously.

Although the potential benefit of biomarker use has been repeatedly addressed, many studies have also found the use to be limited and fraught with uncertainty. One of the biggest problems faced by the biomarker concept has been a shift in management focus from point-source to non-point-source pollution. The biomarker concept was initially conceived for use in point-source studies, and suites of biomarkers were often tested against gradients leading away from point sources. Biomarkers validated in this manner, however, were then used frequently in environments of mixed inputs and diffuse sources, often leading to confusing and contradictory results in complex environmental situations; consequently, it is recommended that the limitations of biomarkers also be considered in any study to avoid augmenting uncertainty within risk calculations.

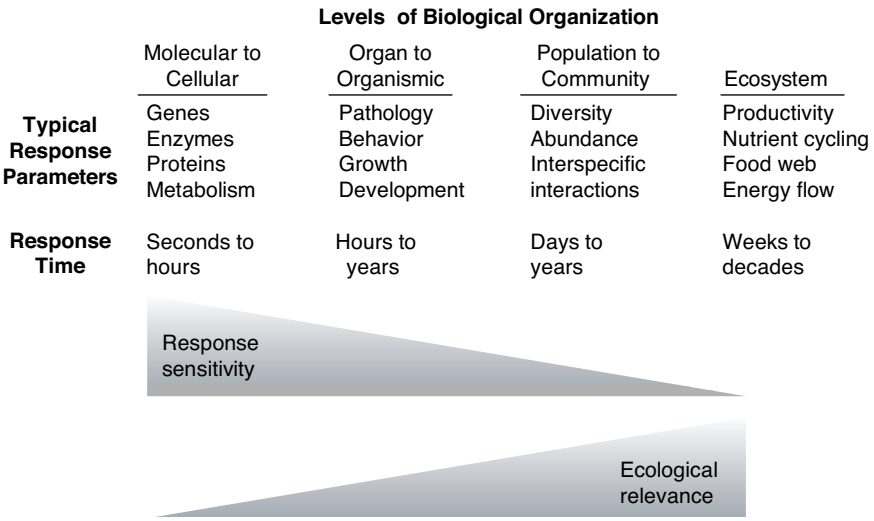

FIGURE 16.1 Relationship of response time, response sensitivity, and ecological relevance with typical response parameters associated with select levels of biological organization.

Hierarchy of Response

With recent method development in -omics, the development and use of biomarkers have primarily occurred at the molecular or cellular level of biological organization. Certainly, organ- and organismic-level responses have been utilized, but most of the emphasis has been focused on biochemical biomarkers, such as alterations in biochemical composition of tissues and body fluids; however, in assessing the adverse effects of environmental chemicals on aquatic ecosystems, the aquatic toxicologist is challenged with evaluating the impact of chemicals at several levels of biological organization. It is recognized that response parameters at each biological level have inherent strengths and weaknesses (Figure 16.1). Molecular biomarkers are valuable because chemicals initiate adverse effects by altering molecular components of the cell, leading to adverse effects on metabolism. These effects may be readily detected in a limited period of time and the biological response manifested at a low concentration of exposure; however, molecular-level responses may not have readily apparent ecological relevance. On the other hand, population- or community-level responses, such as diversity or abundance, have a great deal of ecological relevance but a limited degree of response sensitivity. An additional consideration with respect to response sensitivity is that by the time adverse effects in such parameters as diversity and abundance are detected, significant ecological damage may have occurred. One of the benefits of the biomarker approach is the identification of early-onset changes, which predict increased risk of adverse effects following exposure to environmental chemicals. The predictive value of the biomarker approach should not be overlooked.

Biomarkers have been considered for use to assess higher level biological effects such as fidelity of populations and communities. To predict consequences for individual organisms, populations, and communities by extrapolating from molecular or cellular biomarker responses is difficult. Munkittrick and McCarty (1995) noted that toxicological studies have traditionally been mechanistically focused, attempting to develop an understanding of the interactions between chemical availability and physiological responses of aquatic organisms. This has led to the development of epidemiological techniques, reductionist approaches, and cause-and-effect studies utilizing biomarkers; however, in reality, due to the ecological complexity of stressed ecosystems, environmental monitoring studies must consider diverse sets of stressors involving chemical impacts and habitat alterations as a result of land-use patterns. Ecological studies then have traditionally been descriptive and have attempted to develop an understanding of the interaction of aquatic organisms with their habitat. The effective use of the biomarker approach

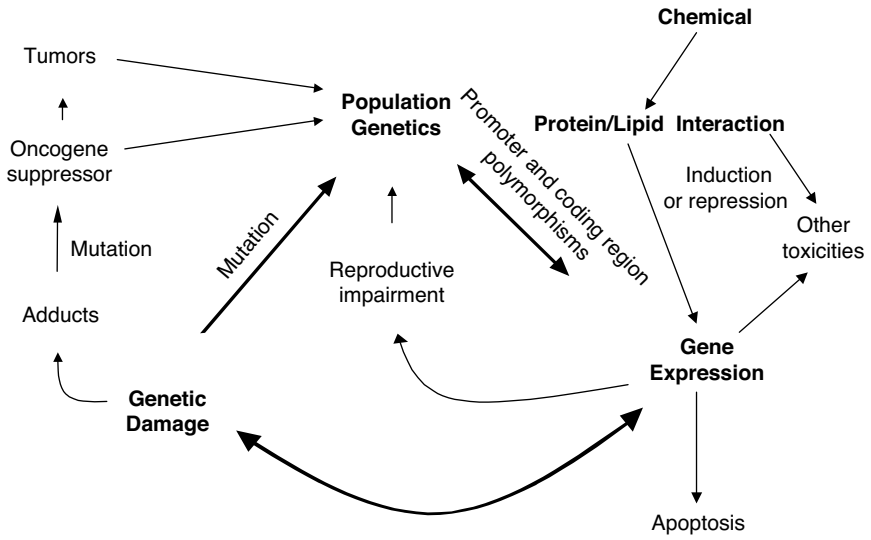

FIGURE 16.2 Interrelationships of biochemical biomarkers.

in environmental monitoring is limited by the inability to extrapolate across the levels of biological organization. The key is the lack of predictability from cause to effect at succeeding levels and the absence of known linkages between cause and effects between various levels of biological organization. In many areas of toxicology, without a knowledge of the cause-and-effect relationship, it is not possible to reliably extrapolate effects between levels of biological organization. Depledge (1994), however, suggested that the use of ecosystem-level responses also may be misleading with respect to identification of the cause-and-effect linkage. Schindler (1987) presented an example in which an experimental lake was artificially acidified, and changes in selected ecological parameters were subsequently followed for several years following. Alterations in lake transparency were initially attributed to acid effects, but later analysis indicated that much of the change was due to unusually low rainfall over the period in which the research was conducted. This provided another example in which correlation was not causation. Clearly, to avoid misinterpretation of biomarker responses, mechanistic links by which chemical effects at one level of organization give rise to detrimental effects at higher levels of biological organization must be established. As an example, alterations in steroid metabolism resulting in changes in hormone profiles which, in turn, alter sexual behavior and the reproductive competence of a population might enable prediction of population-level consequences. It is implicit in such an approach that higher order responses (development and reproduction) would be predicted from measured molecular or cellular level responses. In the design of biomarker strategies, an integrated approach should be considered in which a hierarchy of responses are evaluated. The hierarchy can be constructed based on the level of biological organization that is being monitored or on different degrees of response sensitivity; in fact, as indicated in Figure 16.1, these hierarchical approaches are parallel.

Biochemical Biomarkers

Alteration of biochemical defense systems is typically the initial response to any toxic insult by a xenobiotic; hence, measurement of these systems can be extremely sensitive indicators of altered cell function (Figure 16.2). As discussed above, however, it is imperative that a specific understanding of the normal homeostatic roles for these mechanisms be achieved prior to their use as indicators of exposure, effect or susceptibility. For a discussion and listing of biochemical markers, see Stegeman et al. (1992). For a more thorough discussion of practical uses of biochemical endpoints in aquatic organisms, see

Schlenk and Di Giulio (2002) and Van der Oost et al. (2003). This portion of the chapter focuses on inducible proteins, metabolites, and genotoxic endpoints, but it should be noted that other candidate responses for biomarkers include the inhibition (i.e., via cholinesterases) or repression of specific proteins.

Inducible Proteins

Most biochemical defenses respond to cellular injury by increasing levels of defenses through self-regulating signal transduction mechanisms. These defenses are usually proteins that serve numerous cellular functions, many still unknown. Depending on the dose (or concentration) of the toxicant, these systems are often adaptive; however, when the dose exceeds the capacity of such systems to function properly, irreversible cell injury and toxicity may result. Thus, measuring these systems may provide early warning of danger to the cell as well as help elucidate potential mechanisms of cellular injury.

These particular markers are proteins primarily regulated at the transcriptional level. The protein itself can be measured by enzyme-linked immunosorbent assay (ELISA) or western blot using protein-specific antibodies, many of which are commercially available, or, alternatively, the transcript can be measured by (1) northern blot or ribonuclease protection assay (RPA) using respective cDNA and cRNA probes or (2) quantitative reverse-transcriptase polymerase chain reaction (qPCR) using conserved nucleotide sequences found in several species. If the protein has enzymatic activity, then an additional measure of catalytic activity might be added to one or two of the above. Of the three methods, measurement of protein content is typically the most robust, as activity and transcript levels can be easily degraded by excessive temperature or by nonspecific ribonucleases, respectively. Although -omic studies have already led to the discovery of several other transcriptional biomarkers of toxicity, the discussion here focuses specifically on a few endpoints that have been more well-characterized in fish. (*Note:* Vitellogenin is not discussed in this chapter, as it is described in other chapters within this text.)

CYP1A

Cytochrome P450 monooxygenases (CYPs) are a multi-gene family of enzymes that occur in nearly all plants and animals. These enzymes carry out an array of reactions, and before the recent identification of individual isoforms and their substrate specificities P450s were known collectively as the *mixed-function oxidases*. Because of the plethora of genes that encode these proteins, a nomenclature committee has been appointed to classify each protein into specific gene families (http://drnelson.utmem.edu/nelsonhomepage.html). One of the most common and highly conserved is the CYP1A subfamily. Excellent reviews have been published on the natural history, function, and regulation of this subfamily of enzymes and their potential use in biomonitoring (Bucheli and Fent, 1995; Stegeman, 1993; Stegeman and Hahn, 1994; Stegeman and Lech, 1991; Van der Oost et al., 2003). CYP1A expression is increased primarily through activation of a cytosolic receptor known as the aryl hydrocarbon (Ah) receptor, which eventually serves as its own transcription factor initiating CYP1A mRNA expression. Ah receptors have a relatively selective binding region and prefer planar aromatic hydrocarbons as agonists (Hahn et al., 2005); thus, expression of CYP1A, which has a typically low basal expression rate, can be used as a biomarker of exposure to various planar aromatic hydrocarbons ranging from polychlorinated biphenyls (PCBs) and dioxins to numerous polycyclic aromatic hydrocarbons (PAHs).

Although most of the metabolites resulting from CYP1A-catalyzed reactions are hydroxylated derivatives of parent compounds, the reaction often involves the formation of reactive electrophilic intermediates that may undergo nucleophilic attack by critical macromolecules such as sulfhydral groups of proteins and amine moieties of nucleic acids. Because of the enhanced possibility of protein or DNA adduction, which may result in cellular dysfunction, induction of CYP1A has been proposed to be used as a biomarker of effect. Studies have shown relationships between CYP1A expression and reproductive alterations following exposure to PCBs or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Cook et al., 1997; Teraoka et al., 2003). CYP1A catalytic activity in embryos and eggs has been used to determine toxicity equivalency factors (TEFs), which allow calculations of threshold values for dioxin-like compounds to adversely affect development in eggs of salmonids (Cook et al., 1997). These data suggest that CYP1A expression may be used as biomarker not only of effect but also of susceptibility.

Expression of CYP1A is extremely variable among individuals, and some organisms have been shown to induce multiple isoforms from this family. Numerous allelic variants have been observed in an inbred strain of rainbow trout (Buhler and Wang-Buhler, 1998), as well as wild Atlantic tomcod (Wirgin et al., 1991), which not only complicates nomenclature but may also have significant implications for biomarker studies. For example, evaluation of tomcod recalcitrant to CYP1A induction in PCB-contaminated sites would potentially lead to an underestimation of exposure (Yuan et al., 2001). Although it has been argued that induction of defenses normally provides selective advantages (Barrett et al., 1997), several field studies have shown phenotypic and genotypic evidence that chronic exposure to Ah receptor agonists may provide selective pressure to somehow repress CYP1A induction. So, a question that arises when considering CYP1A as a biomarker of susceptibility is do animals that have impaired expression of CYP1A have a selective advantage over animals that have normal inducibility of CYP1A? In addition, what if measurements are performed on subpopulations in which CYP1A induction is impaired? Significantly, in every case where a subpopulation of fish has been identified as having a repressed CYP1A response, overt signs of contamination were present that would probably be noted by other mechanisms of observation (i.e., absence of biomass); however, research examining these issues is necessary to validate and interpret biomarker data obtained from CYP1A.

Because CYP1A is an enzyme, activity is typically measured using the substrate ethoxyresorufin which is *o*-deethylated by ethoxyresorufin-*O*-deethylase (EROD) to a fluorescent product, resorufin, which can easily be measured. Because EROD activities are generally measured using liver homogenates that also tend to accumulate numerous CYP1A substrates, activity may be inhibited by residual substrates or metals. Thus, CYP1A protein or mRNA should also be measured to validate negative EROD results, especially when animals are taken from sites known to be heavily polluted by classical CYP1A substrates such as PCBs, PAHs, or metals.

To maximize signal-to-noise ratios, it is suggested that male or sexually immature animals be used to conduct studies. These animals should be of the same size and developmental stage. CYP1A as well as most inducible protein systems within the cell are greatly affected by circulating hormones; for example, CYP1A is typically downregulated by estradiol, so signal-to-noise ratios are normally reduced in sexually mature females when circulating levels of estradiol are enhanced (Stegeman and Hahn, 1994). In conclusion, although CYP1A has demonstrated strong correlations with exposure to particular planar aromatic hydrocarbons, elevated expression should not be equated with toxicity. Induction demonstrates Ah receptor activation which may or may not lead to toxicity.

Metallothioneins

Whereas CYP1A provides information regarding planar organic chemicals, metallothioneins (MTs) are typically induced in response to metal exposure. Found in large concentrations in the liver, MTs are low-molecular-weight cytosolic proteins of which 30% of the residues are cysteine. Because of this large thiol concentration, MTs have the ability to bind many transition metals and act as free-radical scavengers. Although expression of MT is transcriptionally upregulated in response to exposure to metals, induction is also observed as part of the generalized positive acute protein stress response associated with cellular injury (Hamer, 1986). Thus, MTs can also be induced by extracellular inflammation, starvation, and oxidative stress resulting from disease or chemical exposures (Sato and Bremner, 1993). Although probably involved secondarily in metal homeostasis, the function of MT is still a mystery.

Metallothioneins have been isolated from numerous fish species and have been extensively studied in trout species (see reviews by Kling and Olsson, 2005; Olsson, 1993, 1996; Roesijadi, 1992). Several field studies have indicated that MT expression in adult organisms strongly correlates with transition metal content from metal-contaminated sites (Farag et al., 1995; Hogstrand et al., 1991; Kaplan et al., 1995); however, other studies have failed to demonstrate relationships primarily due to influences from non-metal inducers, sexual development, or seasonality (Rotchell et al., 2001; Stegeman et al., 1992). Most controlled laboratory studies with fish have indicated induction of MT following exposure to most transition metals that have shown inducibility in mammals (i.e., cadmium, copper, zinc) (Kling and Olsson, 2005; Olsson, 1993, 1996; Roesijadi, 1992). As with mammals, there appear to be significant developmental and gender-related differences in MT expression within fish (George et al., 1996a,b;

Olsson et al., 1995). Regarding the latter, sexually mature female fish again may be poor choices to conduct biomarker studies with MT because they possess enhanced hepatic and serum concentrations of vitellogenin, the zinc-containing egg yolk precursor protein. Consequently, in certain species such as trout, MT levels tend to be repressed by estradiol in sexually mature females (Olsson et al., 1995). This, however, does not appear to be the case in channel catfish, which did not show any statistical differences in MT levels between sexes following chronic (10-week) copper exposures (Perkins et al., 1996).

As mentioned above, other factors also tend to induce MT, especially physiological processes that induce cytokine production or oxidative damage, such as inflammation or general stress. Many of these processes related to general stress also involve the redistribution of metals as well; for example, induction of cortisol tends to cause a redistribution of zinc and copper to the liver which has been shown to induce MT expression (Schlenk et al., 1998). Because MT can be an indicator of acute as well as chronic stress, it is imperative that other acute-phase proteins be measured to verify an acute stress condition (see later discussion on heat shock proteins). Moreover, handling of animals should be minimized to enhance the signal-to-noise ratio between exposed and control samples. This is especially important when conducting cage studies, where animals should be maintained for periods to allow acclimation to occur.

Several investigators have indicated that simple tissue residue measurements of metals would provide the same information as MT and be a better indicator of exposure and possibly effect. A study of channel catfish by Perkins et al. (1996) compared various whole-animal endpoints such as body length, weight, liver weight, and condition factors to hepatic MT expression and copper content after a 10-week exposure to copper. They found that MT protein expression had a significantly stronger correlation to each endpoint than hepatic copper content; in addition, strong correlations were observed between lipid peroxidation and MT in trout chronically exposed to zinc and copper (Farag et al., 1995). These data suggest that MT may also be useful as a biomarker of effect, but only effects mediated by MT-binding metals. Studies examining the effects of low-level arsenical exposure demonstrated dose-dependent increases in MT expression in channel catfish but failed to deplete hepatic glutathione or induce lipid peroxidation (Schlenk et al., 1997). Conversely, studies performed in PLHC-1 cells showed a direct correlation between glutathione depletion and MT induction following cadmium exposure (Schlenk and Rice, 1998). Reasons for this discrepancy are unclear, but future studies with nonbinding inducers may help us to better understand the functional roles of MT and promote its use as an indicator of effect. For discussion about the potential uses of MT as a biomarker of exposure and effect, see Stegeman et al. (1992). For discussions regarding measurement methods, see Schlenk and Di Giulio (2002).

Few studies have examined the role of MT as a biomarker of susceptibility. Several groups have examined species differences in MT expression and have shown that fish with higher basal concentrations or who are more efficient inducers tend to be protected from metal toxicity (Kille and Olsson, 1994; Kille et al., 1991). Benson and Birge (1985) demonstrated that fish residing within metal-contaminated environments had higher MT levels than the same species in a reference pond and that the animals from the metal-contaminated site were more resistant to cadmium and copper. Genetic knockout (MT-null) mice not only are more susceptible to metal toxicity and oxidative stress (Masters et al., 1994) but are also obese, indicating involvement in energetics or basal metabolism (Kling and Olsson, 2005). Clearly, more studies are necessary to determine whether individuals within populations may be more or less susceptible to toxicity based on their ability to express MTs.

Stress Proteins

Stress proteins, originally known as heat shock/stress proteins (HSPs), are a nonspecific group of positive acute-phase proteins that serve several protective and homeostatic functions within the cell. This discussion here focuses primarily on HSP70, HSP30, HSP60, and HSP90. The number and exact size of heat shock proteins are specific to both tissue and species. Except for the highly inducible proteins of the HSP70 family (specifically, HSP72), all of these proteins are present in low concentrations under normal conditions in most organisms studied (Iwama et al., 1998; Sanders, 1990, 1993). Proteins of the HSP70 and HSP60 families are primarily chaperone proteins that are upregulated during proteolysis and aid cells in the folding and transport of newly synthesized proteins. They have been characterized in several fish species and consistently respond to numerous organic and inorganic chemicals (Iwama et

al., 1998; Sanders, 1990, 1993). While HSP70s can be found in the cytoplasm and nucleolus, HSP60 (chaparonin) is a mitochondria protein (Schlesinger, 1994). Each is highly conserved, and antibody as well as nucleotide probes to mammalian forms have been shown to recognize homologous forms in numerous fish and invertebrate species (Dyer et al., 1991, 1993; Iwama et al., 1998; Sanders, 1993; Stegeman et al., 1992). The cytosolic HSP90 protein is an integral part of several receptor complexes (i.e., Ah and steroid receptors) and is induced in catfish, medaka, and fathead minnows following exposure to detergents and PAHs (Villalobos et al., 1996). Other studies observed induction in rainbow trout following arsenite exposure (Kothary and Candido, 1982). Unlike the other HSPs discussed in this section, HSP30 actually serves an enzymatic function known as heme oxygenase, which catabolizes prosthetic groups to biliverdin, which is then converted to bilirubin by biliverdin reductase (Stegeman et al., 1992). Induction of HSP30 protein and activity as a result of chemical exposure have been observed in only relatively few fish species (Ariyoshi et al., 1990; Brown et al., 1993; Grosvik and Goksøyr, 1996; Kothary and Candido, 1982; Sanders, 1993; Schlenk et al., 1996a). In most of these studies, consistent induction was observed following exposure to cadmium or arsenite; however, exposure to phenylhydrazine and high lipid diets appears to increase concentrations of HSP30 in Atlantic salmon (Lunde et al., 1998). Because HSP30 is involved in catabolism, induction of HSP30 has been shown to be inversely proportional to CYP1A and total CYP activity (Schlenk et al., 1996b; Stegeman et al., 1992); thus, HSP30 may prove to be a confirmatory marker of CYP repression.

With the exception of the HSP70 family of proteins, little work has been done to calibrate or characterize these proteins in fish; hence, caution should be used in evaluating data using these proteins alone without other better characterized systems (Kohler et al., 2001). In laboratory studies with rainbow trout, HSP70 levels in juveniles were significantly increased in gills of juveniles exposed to a mixture of cadmium, copper, lead, and zinc for 21 days, in both water and food (Williams et al., 1996). This response correlated with decreases in whole-body potassium and increased concentrations of whole-body metals in juveniles (Farag et al., 1994). Interestingly, no relationship was observed in livers of juveniles or any tissues of adults (Williams et al., 1996). Evidence supporting HSP70 as a biomarker of effect was also observed in β-naphthoflavone (BNF)-treated rainbow trout, in which altered metabolic status of the liver as evidenced by lower phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase, and 3-hydroxy-acyl-coA dehydrogenase activities correlated to hepatic HSP70 expression. This study also showed that HSP70 in this species was not modified by handling stress (Vijayan et al., 1997). Relationships between HSP70 expression and ovarian follicular apoptosis were observed in white sucker exposed to bleached kraft pulp mill effluent (Janz et al., 1997). Seasonal differences in HSP70 expression have also been observed in wild fathead minnows, Atlantic salmon, yellow bullhead catfish, and rock bass, with highest levels of expression being in winter and lowest in summer and fall (Fader et al., 1994).

Compelling evidence suggests that HSP70 may be a useful bioindicator of general cellular stress relating to proteolysis. Although the induced synthesis of the protein is transient, the turnover is much less rapid and the proteins tend to accumulate upon continued cellular stress (Sanders, 1990). In addition, the kinetics of induction appear to be longer following chemical-induced stress compared to heat-induced stress, and recovery is not achieved until several days following exposure to metal, presumably due to accumulation of the metal in the cell (Stegeman et al., 1992). Overall, significant gaps remain in characterizing basal activities of piscine HSPs, especially regarding potential susceptibility, gender-related, and developmental differences. Thus, it is suggested that these proteins be used in conjunction with other acute phase protein markers (i.e., MT) to verify effect.

Multidrug Resistance (P-Glycoprotein)

P-Glycoprotein is a multi-substrate membrane transport protein involved in the resistance of tumor cells to chemotherapy (Juliano and Ling, 1976). Also known as the multidrug resistance (MDR) or multixenobiotic resistance (MXR) mechanism, P-glycoprotein expression has been shown in numerous studies with invertebrates and humans to be regulated by exposure to various classes of organic and inorganic chemicals (Kurelec, 1992, 1997). Because of this ability to be upregulated in response to multiple chemicals, MDR would not be considered to hold promise as a biomarker of exposure to a specific class of chemicals; however, increased expression has been observed, especially in neoplastic tissues, and

may be used as a biomarker of effect (i.e., oncogenic activation) and potentially susceptibility (Kurelec, 1997). The latter was indicated in studies with carp (*Cyprinus carpio*) pretreated with an MDR inhibitor (verapamil) and then treated with various PAHs, resulting in significantly enhanced accumulation of PAHs and CYP1A activity compared to fish that did not receive verapamil (Kurelec, 1997). Similar studies were carried out with invertebrates showing enhanced susceptibility to chemical toxicity when MDR was inactivated or in animals that expressed lower endogenous concentrations (Kurelec, 1992).

Characterizing MDR expression in fish species has been the subject of several recent studies (for a review, see Strum and Segner, 2005). MDR appears to be endogenously expressed in numerous tissues, including renal proximal tubules of *Fundulus heteroclitus* (Miller, 1995), bile canaliculi, exocrine pancreas, lumenal surface of the intestinal epithelium, interrenal tissue, branchial blood vessels, gas gland, pseudobranch, and the gill transverse septa in *Poecilia reticulata* (Hemmer et al., 1995). In cholangiocellular carcinomas of PAH-contaminated winter flounder, expression of hepatic MDR was not observed using immunohistochemistry (Bard et al., 2002); however, significant MDR expression was observed in the intestine of fish from the contaminated site. Overexpression of MDR was observed in homogenates of livers bearing tumors from a population of *Fundulus heteroclitus* from a creosote-contaminated site on the Elizabeth River in Virginia (Cooper et al., 1999). MDR was low in healthy, injured, and extrafocal tissue of cancerous livers from the Atlantic flounder (*Platichthys flesus*) but increased in transitional stages of foci toward the cell types persisting during progression toward carcinomas (Kohler et al., 1998). In other controlled laboratory studies, mutagenic benzo(*a*)pyrene metabolites as well as other xenobiotics were shown to induce expression of MDR in the gut of channel catfish following treatment (Doi et al., 2001; Kleinow et al., 2000). Basal levels of expression were highest in brain, kidney, gills, heart, and intestine of turbot (*Scophthalmus maximus*) (Tutundjian et al., 2002). Results from these studies and ones in invertebrates (Kurelec, 1992, 1997) provide strong evidence that overexpression of MDR, particularly in the intestines of fishes, may be a component of a genetically based mechanism providing a selective advantage in pollution-resistant populations of predatory aquatic organisms. Clearly, because of the discrepancies and variability of expression in animals within the same species, more characterization studies are necessary to further develop this nonspecific indicator of effect and susceptibility in fish.

Phase II Biotransformation Enzymes

Following initial phase I transformation, which either exposes or adds single polar atoms (i.e., OH) to enhance water solubility and excretion, phase II processes tend to augment this process by adding large endogenous polar molecules (Figure 16.3). Expression of several phase II enzymes in mammals is regulated via the xenobiotic response element (XRE) and thus would be expected to be induced by planar aromatic hydrocarbons and serve as potential verification for exposure to Ah receptor agonists. Examples in fish include uridine diphosphate (UDP)–glucuronosyltransferase (UDPGT), at least one isoform of which is induced by treatment of several Ah receptor agonists such as PAHs and PCBs (Forlin et al., 1996; George, 1994). Studies in the field appear to verify laboratory studies with a direct relationship between UDPGT activity in fish that accumulate PCBs or organochlorines (Van der Oost et al., 2003). In eelpout (*Zoarces viviparous*), seasonal variation in responses were much larger in phase I enzymes than in UGT (Ronisz et al., 1999). For a more in-depth discussion regarding purification and regulation, see George (1994) and Chapter 4 of this text.

Another phase II family of enzymes that can be upregulated following exposure to planar aromatic hydrocarbons in mammals is the glutathione *S*-transferase (GST) family. In fish, the expression of GST activity (chlorodinitrobenzene [CDNB] dehalogenation) in response to acute doses of Ah agonists does not appear to result in significant elevation (George, 1994); however, if fish are chronically exposed to PCBs, GST activity has been shown to be significantly elevated (Forlin et al., 1996). Indeed, numerous contradictory field studies have shown induction, no change, and in some instances repression of activity at various polluted sites (for a review, see George, 1994). This inconsistency in response may be related to the relatively simple assay for GST that has been used in a majority of these studies and which does not differentiate between major GST isoforms (with the exception of the class of GSTs that lack CDNB activity). In fact, in studies characterizing the effects of various inducers on GSTs of plaice (*Pleuronectes platessa*), expression of a GST gene structurally homologous to a class isoform was repressed by planar

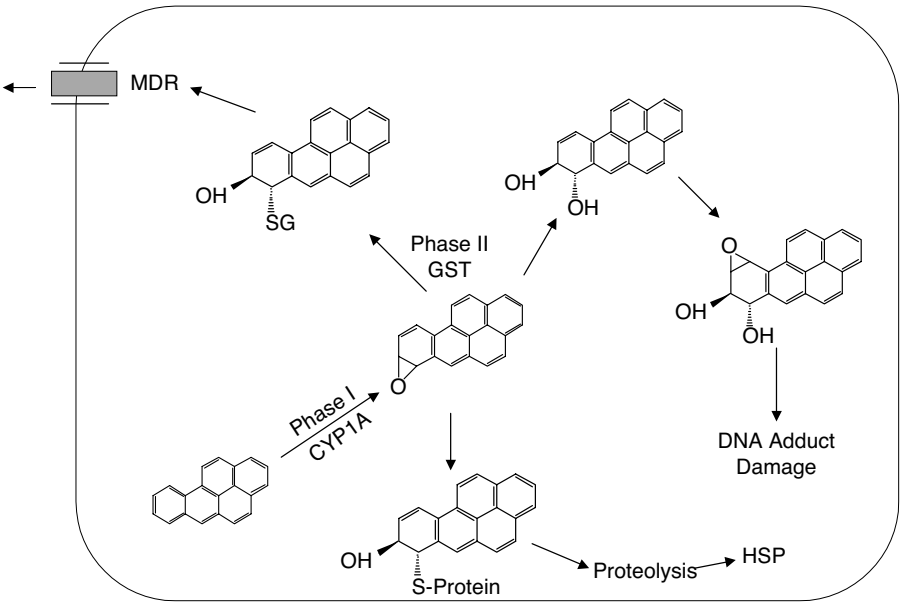

FIGURE 16.3 Relationship between phase I and phase II biotransformation and cellular responses.

aromatic hydrocarbons but induced by various cellular and xenobiotic oxidants (Leaver and George, 1998; Scott et al., 1992). In this way, it is quite likely that planar aromatic compounds, such as PCBs, may induce certain forms of GST expression bifunctionally—through the Ah receptor (xenobiotic response element) or oxidative stress (antioxidant response element) (Forlin et al., 1996). Thus, the use of GST as a biomarker of exposure or effect may be limited unless specific assays and probes of well-characterized isoforms are used together to help understand the relevance of induction or repression.

Many studies in humans have shown that expression of GSTs from the μ subfamily may have dramatic effects on the susceptibility to various cancers (Seidegard et al., 1986, l990). In addition to proteins such as MDR, neoplastic tissue has also been shown to overexpress various μ-class GSTs. In fish, this relationship has not been as strong as that observed with MDR. Few studies examining GST activity (CDNB conjugation) in hepatic lesions have documented consistent relationships (Kirby et al., 1990). In a study of resistant *Fundulus heteroclitus* from a highly creosote-contaminated area in the Elizabeth River, it was demonstrated that this population of fish had significant (sixfold) elevations in hepatic GST activity and protein (Van Veld et al., 1991); however, unlike MDR levels, expression of GST did not seem to differ between neoplastic or normal tissue.

Although UDPGT and GST may be used in certain instances to verify exposure to Ah receptor agonists, their use as biomarkers of exposure is secondary to their use as biomarkers of effect (oxidative stress) and susceptibility (particularly homologous μ-class GSTs). Clearly, many more characterization studies in other species are required before these latter two uses may be implemented in field experiments.

Antioxidant Enzymes

Because oxidative insult can be an endogenous process that occurs normally in specific areas of the cell (i.e., mitochondria), the cell has evolved numerous defenses to oxidant damage (see Figure 16.2). Quantitatively, cellular thiols such as glutathione serve an extremely important role in maintaining the cellular redox potential during oxidative stress. Several enzymes are involved in maintaining glutathione in the reduced state. Glutathione reductase and the synthesizing enzymes of glutathione are strongly regulated by the redox potential of the cell and have been postulated to be used as potential biomarkers of oxidant damage and potentially of susceptibility. Channel catfish, for example, are significantly more resistant to the pathological effects of oxidative stress than bullhead and contain significantly higher

levels of not only glutathione but also GST, glutathione reductase, and other antioxidant enzymes such as DT diaphorase and catalase (Hasspieler et al., 1994).

In addition to oxidant stress, hepatic DT diaphorase is also upregulated in response to Ah receptor agonist exposure, indicating a bifunctional regulatory mechanism for the enzyme. Similar to GSTs, expression following Ah receptor activation is not nearly as pronounced as that of CYP1A (Lemaire et al., 1996); however, because of its bifunctional regulation, its use as a biomarker of exposure must be in combination with either other Ah-receptor-mediated responses (i.e., CYP1A) or oxidative-stress-mediated responses (i.e., GSH depletion or oxidation). Comparative studies of DT diaphorase in seven marine and five freshwater fish species demonstrated a 20- to 100-fold variation between species (Forlin et al., 1995). The enzyme has been shown to be present in turbot (*Scophthalmus maximus*) embryos immediately after hatching, with no change in activity up to 11 days posthatch (Peters and Livingstone, 1996). Chronic exposure of trout to PCBs led to consistent induction (Forlin et al., 1996), and studies assessing expression in the field have observed fairly consistent induction with CYP1A activities and exposure to other planar aromatic hydrocarbons such as PAHs (Livingstone et al., 1992, 1995). Regarding its role as a biomarker of susceptibility, DT diaphorase activity was shown to be lower in brown bullhead, which are not only more sensitive to oxidative damage but also more prone to cancer than other species that possess higher levels of DT diaphorase. Other antioxidant enzymes involved in cellular protection from oxidative damage (superoxide dismutase, catalase, glutathione peroxidase) have not shown any consistent pattern of induction in laboratory or field studies or relationship to toxicity in fish (Stegeman et al., 1992; Winston and Di Giulio, 1991). Although this chapter deals specifically with enzymatic pathways and induction of specific proteins, the interested reader should also evaluate reviews on non-enzymatic endpoints such as GSH/GSSG ratios and lipid peroxidation metabolites (Schlenk and Di Giulio, 2002; Van der Oost et al., 2003). Studies by Regoli and Winston (1998) and Regoli et al. (2000) have also demonstrated some success in utilizing nonenzymatic endpoints, known collectively as total oxyradical scavenging capacity (TOSC).

Endogenous Metabolites

Metabolites in the context of biomarkers are defined as modified endogenous molecules. Certainly it would be possible to measure biotransformation products through advanced analytical chemistry; however, these endpoints are grouped according to the common characteristics of being modified endogenous molecules. PAHs, for example, tend to undergo phase II conjugation with subsequent biliary elimination (see Figure 16.3); hence, fluorescent aromatic compounds (FACs) are actually derived from conjugates (glucuronides, sulfonates, and glutathione adducts) that are modified endogenous molecules. DNA adducts could also be included in this group, but other DNA lesions fail to meet this criteria; consequently, a separate section on genotoxic endpoints has been included below.

Fluorescent Aromatic Compounds

The toxicity of numerous aromatic hydrocarbons, especially PAHs, is generally mediated subsequent to an oxidative transformation of the parent to a reactive intermediate that tends to covalently bind critical macromolecules in the cell, altering normal function. Aromatic hydrocarbons are typically biotransformed and excreted through the bile as oxidized conjugates of the original parent in most vertebrates (Parkinson, 1996). Because the toxicity of aromatic hydrocarbons is often directly related to the metabolism of the compounds, it was suggested that measurement of aromatic hydrocarbon metabolites might be an appropriate indicator of exposure and effect (Krahn et al., 1984). Since this study, this assay has been utilized in various fish species as a biomarker of exposure to aromatic hydrocarbons (Aas et al., 2000, 2001; Collier et al., 1995, 1996; Gagnon and Holdway, 2002; Ruddock, et al, 2000). Studies conducted with English sole (*Parophrys vetulus*) from Eagle Harbor in Puget Sound (French et al., 1996) and oyster toadfish (*Opsanus tau*) in segments of the Elizabeth River in Virginia demonstrated direct correlations between PAH concentrations in sediment, PAH–DNA adducts, CYP1A, and FACs (Collier et al., 1995). In addition to showing relationships with PAH exposure, higher level adverse effects were also observed to correlate with FACs in some instances. Johnson et al. (1992) observed elevated FACs as well as

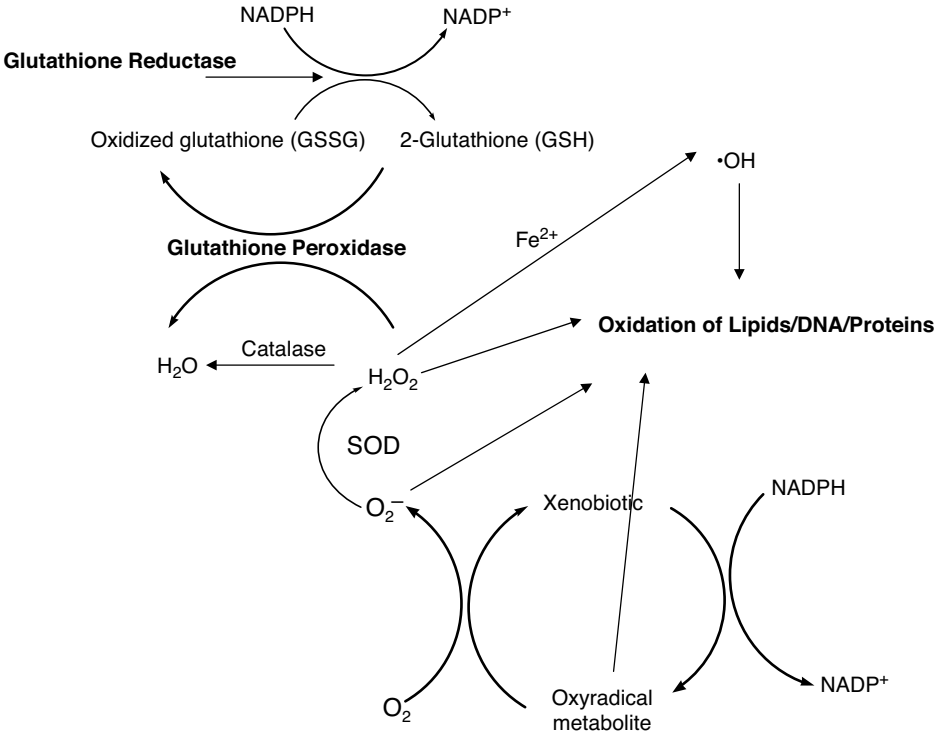

FIGURE 16.4 Pathways for generation and detoxification of reactive oxygen species within cells.

CYP1A in female English sole from Puget Sound that had inhibited ovarian development. In Atlantic flounder caged in contaminated waterways in Norway, a direct correlation was observed between FACs, CYP1A, and serum aspartate aminotransferase, indicating liver damage (Beyer et al., 1996). However, in several fish species from Prince William Sound in Alaska following the *Exxon Valdez* oil spill, no correlation was observed between DNA adducts and FACs, although positive relationships were observed with CYP1A expression (Collier et al., 1996). In fact, in studies with juvenile turbot that were exposed continuously for 15 days to 2 mg/L of crude oil, FACs proved to be a more sensitive indicator of PAH exposure than CYP1A activity (Borseth et al., 1997). One of the few limiting factors in carrying out FAC analyses is the size of the animal, as it is preferable to be able to access the bile easily from captured or sampled fish. It should also be noted that values should be expressed as raw concentrations in animals of similar size. If species or site comparisons utilize animals of differing size, then it has been suggested that values be normalized to biliverdin concentrations in the bile (Richardson et al., 2004). Analysis of these metabolites is possibly one of the most specific indicators for exposure to aromatic hydrocarbons.

Porphyrins

Porphyrins are degradation products of heme catabolism that provide oxyradical scavenging capacity (Figure 16.4). In contrast with other studies with birds and mammals where the measurement of porphyrins has been shown to correlate with exposure to chlorinated hydrocarbons, few studies have been performed in fish (Melancon et al., 1992). Carboxylated porphyrin profiles have been proposed as a biochemical indicator in lake whitefish (*Coregonus clupeaformis*) exposed to bleached kraft pulp mill effluent (Xu et al., 1994). In one other study, uroporphyrinogen decarboxylase of pike (*Esox lucius*) collected from a site of the Rhine River heavily contaminated with aromatic organohalogen compounds was lower than in pike from the river Lahn, which did not have elevated organohalogen levels (Kloss, 1986). In addition to inhibitory effects on uroporphyrinogen decarboxylase, *in vitro* studies using fish hepatoma cell lines have demonstrated that halogenated aromatic compounds may also disrupt porphyrin

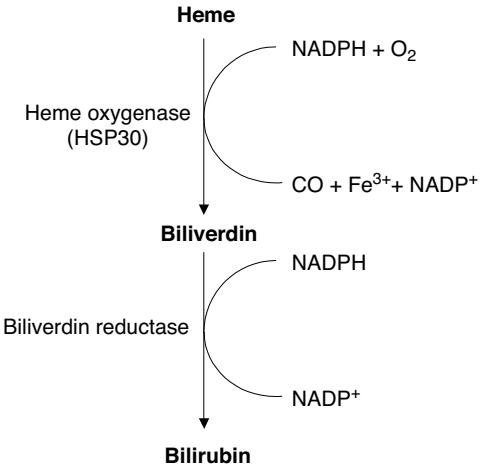

FIGURE 16.5 Degradation pathway of heme.

metabolism through a mechanism involving interaction of CYP1A with the halogenated aromatic compound potentially initiating oxidative stress (Hahn and Chandran, 1996). Based on the relationships between HSP30 (oxygenase), CYP1A (protein), bilirubin, and biliverdin (endogenous Ah agonists) (see Figure 16.5), assessment of porphyrin profiles may provide significant mechanistic insight when they are measured with these other proteins and metabolites. It is obvious that the use of these metabolites as biomarkers has been largely overlooked in fish and deserves further characterization.

Retinoids

Vitamin A-derived compounds, also known as retinoids, are an additional group of endogenous metabolites that have shown promise as biomarkers of effect and potential exposure to planar halogenated aromatic compounds when used in combination with other markers (Alsop et al., 2005; Arcand Hoy et. al. 1999; Peakall, 1992; Spear et al., 1992). In brook trout (*Salvelinus fontinalis*), a single injection of 5 μg/g of a coplaner PCB (3,3′,4,4′-TCBP) reduced retinoids in several tissues (Ndayibagira et al., 1995). In the same study, lake sturgeon (*Acipenser fulvescens*) collected from a contaminated waterway near Montreal, Canada, had significantly lower retinoid levels in the intestine. In addition, field studies with white sucker demonstrated a direct relationship between maternal retinoid loss, CYP1A activity in the liver, and prevalence of embryonic malformations in animals collected from the same contaminated location, with significant gender-related differences in hepatic retinoid expression (Branchaud et al., 1995). Following a chronic exposure of 3 years to PAH-contaminated sediment in a mesocosm, retinol concentrations in plasma and liver were significantly reduced in Atlantic flounder (Besselink et al., 1998). In fact, a negative, nonlinear correlation was found between retinol concentrations and CYP1A expression, indicating that retinoids may be a significant indicator of exposure and effect of planar aromatic compounds or other Ah receptor ligands (Alsop et al., 2005). Due to the meager research efforts directed toward retinoid metabolism and disposition in fish, the relationships between altered hepatic retinoid levels and fish health are uncertain; however, given the prominent role of retinoids in cellular metabolism and development, relationships appear likely and deserve further study.

Genotoxic Endpoints

Alterations to an organisms' genetic material (DNA) represent an impact of the highest order. The consequences of DNA alterations potentially affect all levels of biological organization, from the molecular to community level. Of central importance to an organism are its survival and reproductive success. Neither is attainable without maintaining the integrity of its genetic material, DNA. At the molecular level, the corruption of the information coded in DNA may not pass beyond the boundary of an affected

TABLE 16.3

Endpoints Used to Assess DNA Damage

DNA adducts
Spectroscopic identification (LC–MS)
Base oxidations (8-hydroxyguanosine)
^{32}P-Postlabeling
Alkaline unwinding
Comet assay
Micronuclei examination
Flow cytometry
Specific evaluations of point mutations

TABLE 16.4

Methods to Evaluate Genetic Effects in Populations of Organisms

Allozymes
Mitochondrial DNA
Randomly amplified polymorphic DNA (RAPD) techniques
Amplified fragment length polymorphisms (AFLPs)
DNA fingerprinting
Microsatellites

Source: Theodorakis, C.W. and Wirgin, I.I., in *Biological Indicators of Aquatic Ecosystem Stress*, Adams, S.M., Ed., American Fisheries Society, Bethesda, MD, 2002, pp. 149–186. With permission.

single cell; yet, there exists the potential for a cascade of effects adversely affecting the functioning and survival of cell populations, organs, and the organism as a whole. Furthermore, damage sustained in reproductive cells has the potential to adversely affect fertilization success, development, and the ultimate survival of offspring, thereby causing a net negative affect at the population and community levels (Shugart et al., 1992; Theodorakis and Wirgin, 2002).

Alternatively, toxicological influences may not directly damage an organism's genetic material but can exert selective pressure changing the genetic composition of a population (Anderson et al., 1994; Theodorakis and Wirgin, 2002). A proliferation of methods has allowed the detection of genetic alterations (Theodorakis and Wirgin, 2002; Wirgin and Theodorakis, 2002). These methods measure genetic effects at all levels of biological organization but can be broadly characterized as those that measure structural damage at the molecular/cellular level (Table 16.3) and those that measure changes in the genetic composition of individuals in a population (Table 16.4). In the latter case, investigators are studying whether genetic diversity or population genetic composition can serve as an indicator of environmental quality (Anderson et al., 1994; Bickham and Smolen, 1994; Gillespie and Guttman, 1993; Roark et al., 2005a,b; Xie and Klerks, 2004). Numerous studies have explored whether exposure to pollutants can result in the selective survival of specific genotypes and whether observed shifts in the genetic composition of a population can be utilized to assess environmental quality or determine the potential of a population to survive various insults. Several studies have demonstrated the correlation of fish population survivorship with increased frequencies of specific alleles at various loci (Nadig et al., 1998; Schlueter et al., 2000; Sullivan and Lydy, 1999; Theodorakis et al., 1998). In most cases, genetic variability is determined by the extraction and electrophoretic differential mobility of enzymes or DNA segments produced using the randomly amplified polymorphic DNA (RAPD) technique. In studies of various polymorphic loci, the frequencies of several genotypes have been found to be significantly different in the surviving population than in those of the initial population after exposure to some stress, usually a chemical stress (Newman et al., 1989; Roark and Brown, 1996; Schlueter et al., 1995, 2000; Silbiger et al., 2001; Sullivan and Lydy, 1999). It is generally believed that average genetic heterozygosity enhances fitness by increasing

the diversity of enzyme products which confers a broader biochemical tolerance to a variety of environmental conditions; however, this has not always turned out to be the case and is dependent on the species, number of loci examined, and direct or indirect mechanistic linkage of their products to the applied stress or stressors (Schlueter et al., 2000).

The RAPD assay measures differences in the genetic structure of individual animals held under varied conditions. Isolated DNA is digested, and, using various primers, fragments of the digested DNA are amplified via the polymerase chain reaction (PCR). The resulting product is electrophoretically separated on the basis of size. Comparisons are made of the resulting banding patterns, comparing relative position and the presence or absence of bands. The long-term goal of this approach is to develop probes to bands that appear to be sensitive to contaminant exposure. The presence or absence of a band does not reveal functional information, the identity and significance of a potential marker band has to be established. This can be accomplished by characterizing potential bands using probes produced from subsequent cloning and sequencing steps. For both approaches, a great deal of work is still necessary to define the conditions affecting these population-level measures and the mechanisms by which this differential survival is expressed.

The cell is a protective barrier surrounding the DNA that presents physical barriers in the form of the plasma and nuclear membranes that are interspersed with protective enzyme systems geared toward intercepting potentially damaging agents. Even the manner in which the DNA molecule is tightly wound is in part a strategy for defense, as it restricts access to the bulk of the material from harmful influences. Yet, damage occurs due to the influence of normal background radiation or as a result of normal cellular functions, such as the structural demands of relaxation and presentation of the molecule for reading or replication or by interaction with damage-inducing metabolic byproducts such as free radicals. It has been estimated that chromosomal DNA sustains approximately 85,000 alterations per day without a loss of function in the form of base alterations, adduct formation, strand breaks, and cross linkages (Bernstein and Bernstein, 1991). These numbers will, of course, vary in different types of cells, but these influences are a given and are efficiently dealt with by cellular DNA excision-repair enzymes. Beyond these various barriers, defenses, and repair mechanisms lies a last line of defense, which restricts the survival or replication of potentially corrupted genetic information, halting cellular propagation by cell directed death or apoptosis.

Various molecular/cellular methods have been developed for detecting DNA damage of different types, DNA adducts, DNA base mutations at sensitive sites in the genome, and DNA strand breaks. Certain genomic sites sensitive to chemically induced alterations such as tumor suppressor genes and oncogenes have been shown to contribute to the initiation and progression of cancer (Bailey et al., 1996; Cachot et al., 2000; Greenblatt et al., 1994). Monitoring of these genomic sites may provide sensitive biomarkers of mutagen exposure and the early onset of carcinogenesis. Examination of K-*ras* exon 1 from aflatoxin B_1- and PAH-treated rainbow trout embryos revealed mutations at codons 12 and 13 (Bailey et al., 1996). Sequence data and 3′ mismatch assay examination of oil-treated and non-oil-treated embryos of pink salmon (*Oncorhynchus gorbuscha*) also identified mutations at both codons 12 and 13 in the oil-treated population exclusively (Roy et al., 1999). These results are encouraging, but before this approach can be utilized for routine environmental monitoring many questions remain, such as the dose relationship of contaminant exposure and these mutations, the interspecific sensitivity of wild fish populations to induced mutations, and whether these mutations are transmitted intergenerationally.

Exposure to some contaminants can be determined if these compounds or their metabolites are able to bind DNA, forming adducts. PAHs are the most thoroughly studied adduct-forming environmental contaminants. Currently the most sensitive method for detecting DNA adducts is the ^{32}P-postlabeling method (Gupta and Randerath, 1988). Many researchers have detected DNA adducts in liver tissue and blood cells of PAH-exposed fish in laboratory and field collection studies (Akcha et al., 2003; Ericson et al., 1998; French et al., 1996; Lyons et al., 1999; Pinkney et al., 2004). Maximum levels of adduct formation have been shown to be reached in a matter of days and to persist at measurable levels for many weeks after exposure (Stein et al., 1993). In comparisons of the adducts found in wild fish populations and fish exposed to sediment from the field collection sites, similar types of adduct profiles have been resolved, and a dose–response relationship has been observed between PAH exposure and hepatic DNA adduct levels (Collier et al., 1993; Ericson et al., 1998; French et al., 1996). A crucial relationship that illustrates the significance of DNA adduct formation is the co-occurrence of this damage

with increasing frequencies of hepatic lesions (Myers et al., 1998a). Similar relationships have been observed at what is recognized as one of the initial steps of the neoplastic process: CYP1A induction and metabolic activation of adduct-forming PAHs. Many studies have demonstrated the co-occurrence of CYP1A induction and the increase in DNA adducts (Collier et al., 1993; Myers et al., 1998a; Ploch et al., 1998), as well as coordinated reductions in both CYP1A expression and DNA adduct levels in English sole liver neoplasms (Myers et al., 1998b). Adduct detection is currently being used by many laboratories worldwide as a dosimeter of genotoxic exposure.

Genotoxic damage resulting from PAH exposure is not limited to adduct formation. A broad spectrum of contaminants, including PAHs, pesticides, PCBs, dioxins, furans, and trace metals, can generate highly reactive oxygen radicals through a variety of chemical pathways. These radicals can directly damage DNA, resulting in oxidative modification of guanine and adenine bases or DNA strand breaks. Oxidized bases can be misread during replication, resulting in base sequence mutations (Kuchino et al., 1987). Examination of oxidative DNA damage has not consistently revealed a straightforward relationship between contaminant concentration and occurrence of damage (Rodriguez-Ariza et al., 1999). Oxidative damage may be particularly sensitive to tissue specific cellular transport mechanisms, antioxidant defenses, and the DNA repair capacity (Regoli et al., 2003). Of course, such linkages and interactions affecting biomarker concentrations should always be considered when a biomarker of any kind is utilized. Also of concern are procedural influences on damage. *In vitro* studies comparing oxidized DNA base measurements determined by high-performance liquid chromatography (HPLC) and the comet assay have shown that the isolation, storage, and hydrolysis steps used for HPLC methods induce oxidative damage themselves (Collins et al., 1996).

The most prevalent type of genetic damage is the DNA single-strand break. Tens of thousands occur daily in a cell (Bernstein and Bernstein, 1991), and many toxicants have been shown to cause strand breaks in a dose-dependent manner (Tice, 1996). Strand breaks may be introduced directly by genotoxic compounds, the induction of apoptosis or necrosis, oxygen radicals or other reactive intermediates, and the action of excision repair enzymes (Eastman and Barry, 1992; Park et al., 1991; Speit and Hartmann, 1995). Many methods are available for measuring strand breaks. Most rely on the denaturation of cellular DNA followed by some means of enumerating broken strands. Many early methods relied on rates of unwinding as determined by the incorporation of a fluorescent dye in double-stranded DNA or the separation of reannealed double-stranded DNA from break produced single-stranded DNA by centrifugation or filtration (Mitchelmore and Chipman, 1998; Shugart et al., 1992). These and similar methods have been used to demonstrate the dose relationship of DNA strand breakage to applied toxicological insults *in vitro*, *in vivo*, and in field-collected fish (Everaarts et al., 1993; Kosmehl et al., 2006; Mitchelmore and Chipman 1998; Nehls and Segner, 2005; Shugart, 1988; Sugg et al., 1995, 1996).

The comet assay has been used to determine oxidative DNA lesions as well as single-strand breaks, double-strand breaks, DNA repair activity, frequency of apoptosis, and pyrimidine dimer lesions in single cells (Collins et al., 1993; Gedik et al., 1992; McKelvey-Martin, 1993; Tice 1996). An extension of earlier DNA denaturation methods, the comet assay utilizes the electrophoretic mobility of relaxed or broken strands of DNA following denaturation to detect damage. The assay has the added advantages of not requiring DNA extraction and purification from tissues, measuring strand breaks in individual cells, and requiring small sample sizes of ~10,000 cells. Because the method requires such small numbers of cells per sample, it has been used to screen the genotoxicity of various compounds on isolated fish cells *in vitro* (Avishai et al., 2002; Tiano et al., 2000), as well as the dose-dependent antioxidant (protective) properties of various compounds (Villarini et al., 1998). Belpaeme et al. (1998) conducted systematic *in vivo* genomic damage studies on marine flatfish using the comet assay and concluded that the method was simple and sensitive but that care must be taken in choosing protocols and experimental conditions. Using this method, investigators have detected significantly elevated levels of DNA damage in cells of fish from polluted sites compared to reference sites (Hartl et al., 2006; Lee and Steinert, 2003; Pandrangi et al., 1995). Strand damage does indeed appear to be a useful fish biomarker. The current emphasis on alkaline-labile, single-strand breaks limits these methods to the determination of nonspecific damage; however, the comet assay can be modified to specifically express single- or double-strand damage, specific lesions, DNA repair activity, and the cellular presence of photoactive contaminants (Collins et al., 1993; Gedik et al., 1992; McKelvey-Martin, 1993; Steinert et al., 1998b; Tice, 1996).

Due to these capabilities, coupled with the ability of this method to distinguish germ and somatic cells by DNA content and to identify apoptotic cells, the potential exists to develop protocols to identify patterns of damage characteristic of specific inducing agents (Rempel et al., 2006; Roy et al., 2003; Steinert et al., 1998; Tice, 1996).

Physiological Biomarkers

In addition to biochemical assays, the biomarker approach can be applied at a variety of levels of biological organization (Handy et al., 2002a, 2003). Indeed, Peakall (1999) argued that the biomarker concept should relate biological responses at the organism level and below. At the organism level, we should therefore consider physiological and behavioral biomarkers. The notion that physiological or behavioral responses could be used to assess pollutant exposure or effect is not new (Depledge, 1994; Handy and Depledge, 1999); however, in the 1970s and 1980s the practical application of physiological biomonitors was limited by technology that required considerable skill to set up and calibrate and could only record from (at best) a few individual organisms at a time in a laboratory or field-laboratory situation (Cairns and van der Schalie, 1980; Morgan and Kühn, 1984). Advances in electronic technology and our understanding of the effects of pollutants on animal physiology and behavior have made physiological biomarkers a practical proposition (Handy and Depledge, 1999); however, physiological biomarkers do not necessarily have to use high technology, and many simple measurements can be made easily that are informative of exposure or biological effect. Here, we briefly review physiological biomarkers roughly in order of levels of biological organization, starting with measurements in the blood, then organ-level measurements and indices, followed by body system effects (e.g., respiration), and finally integrated whole-animal responses (behavior and animal physiology).

Hematology and Clinical Chemistry

Blood is the most accessible component of the vertebrate body fluid system and has frequently been examined to assess physiological status (Houston, 1997). Many parameters can be measured, including various blood cell counts, whole blood hemoglobin, plasma ion concentrations, and the activity of various enzymes and hormones, as well as concentrations of pollutants in the blood. Questions then arise regarding which blood parameters should be selected as a biomarker and what generic or specific information about exposure or biological effect these blood biomarkers would give the toxicologist.

Blood Sampling Methods

Regardless of the blood parameter to be used as a biomarker, it is essential to (1) standardize blood sampling protocols, and (2) appreciate that many normal biological variables will alter hematology or blood chemistry. This not only is good practice for laboratory work but also becomes essential if the biomarker is to be used in monitoring programs or over long time scales to examine fish populations. Laboratory studies have shown that blood chemistry is influenced by animal handling procedures (Waring et al., 1992) and the use of anesthesia and anticoagulants (Iwama et al., 1989; Korcock et al., 1988), as well as the type and duration of sample storage prior to chemical analysis (Jayaram and Beamish, 1992). Houston (1990) discussed these general considerations for blood sample acquisition, and there is general agreement that blood should be collected within about 5 minutes of initiating capture. Also, if a once-only terminal blood sample is required, then stunning is generally preferable to the use of anesthesia. When handling is prolonged, however, or when other procedures are performed before blood collection (this is not recommended), then anesthesia will reduce the impact of these stresses (Iwama et al., 1989). In the field situation, stunning followed by the collection of caudal or cardiac blood into previously heparinized tubes that are immediately placed in a cooler offers a good chance of collecting a representative blood sample. Every attempt should be made to minimize the time required to capture the fish prior to blood sampling.

Reference values of blood parameters of fish are affected by many biological variables (Hille, 1992; Rogers et al., 2003) that may not be directly related to pollutant exposure or the animal handling issues noted above; for example, blood chemistry can change as the animal grows during chronic exposure

TABLE 16.5

Hematological Endpoints Used as Physiological Biomarkers

Endpoints	Biomarkers
Indices	Hematocrit
	Erythrocyte count/unit blood volume
	Erythrocyte hemoglobin
Blood/plasma ions	Sodium
	Potassium
Blood enzymes, protein, endometabolites	Cholinesterase
	Liver function enzymes (ALT)
	Aminolevulinic acid dehydratase
	Albumin
	Urea/glucose
Hormones	Estrogens
	Androgens (11 ketotestosterone > testosterone)
	Progestins
	Thyroid hormones (T_3/T_4)
	Cortisol
	Insulin-like growth factor

studies and cause time effects in the data that are not associated with toxicant exposure (Handy et al., 1999). Nutritional deficiencies or excesses can alter hematology (e.g., iron status) (Carriquiriborde et al., 2004). General adrenergic or cortisol-driven stress responses that may or may not be associated with toxicant uptake by tissues (Handy, 2003), fish age or strain (McCarthy et al., 1975), season (Houston et al., 1996), and myriad other environmental factors (e.g., stocking density, dissolved oxygen, temperature, salinity) can affect blood parameters. We therefore recommend that the water chemistry, fish body size effects, nutritional status, stocking density, and so on are recorded so the blood biomarker can be interpreted in view of this background variability. Alternatively, variability could be removed during experimental design. Indeed, biomarkers in general should be incorporated into a suite of chemical and other biological measurements if they are to be useful in a regulatory framework (Handy et al., 2003).

Hematological Indices

A list of hematological endpoints is provided in Table 16.5. The hematological variables include the percentage of blood volume consisting of red cells (hematocrit, or Hct), red blood cell count per unit blood volume, and hemoglobin (Hb) concentration. These are the primary indices (directly measured), and a series of secondary indices may be calculated, including mean red cell volume and mean erythrocyte hemoglobin. Houston (1997), however, pointed out that these indices were originally derived for human and veterinary health studies and that error can be introduced into data on fish blood because fish red blood cells have a different shape, membrane flexibility, and erythron profile (percent immature, mature, and dying cells) compared to those in mammalian blood. Houston (1997) suggested that the erythron profile may be the most sensitive parameter in fishes exposed to toxic metals and that Hct is probably the least reliable primary indicator of oxygen carrying capacity. The calculation of secondary indices is not recommended; thus, the erythron profile and perhaps Hb may be the most reliable hematological biomarkers. Blood collected and kept at 0 to 2°C for less than 24 hours can be analyzed for Hct and Hb but may give misleading results for some plasma ions and other metabolites if the whole blood has been stored too long before removing the plasma (Jayaram and Beamish, 1992; Korcock et al., 1988). Alternatively, blood smears can be performed using nonlethal blood sampling and the erythron profile scored (Houston, 1997). Hematology has been performed in field studies on, for example, metal pollution (Haux et al., 1986; Larsson et al., 1985), pulp mill effluent (Oikari et al., 1985; Sepúlveda et al., 2004), wastewater (Hemming et al., 2001), and exposure to PCBs (Everaarts et al., 1993). Similarly, hematology has been used in numerous laboratory studies on pollutants (Berntssen et al., 2004; Handy et al., 1999; Jung et al., 2003; Peuranen et al., 2003; Poleo and Hytterod, 2003).

Blood or Plasma Ions

The effects of pollutants on osmotic and ionic regulation are relatively well known and have been reviewed several times (Evans, 1987; McDonald and Wood, 1993; Wendelaar Bonga and Lock, 1992; Wood, 1992). These effects can include the general loss of electrolytes by diffusion across gills that are damaged by pollutant exposure or specific effects on the absorption and excretion of particular electrolytes (e.g., sodium depletion caused by exposure to copper) (Grosell and Wood, 2002; Grosell et al., 2004; Pelgrom et al., 1995). Blood or plasma ion concentrations have been measured in field situations to yield useful information on toxic effects and the physiological processes involved in survival in unusual water qualities (Haux et al., 1986; Larsson et al., 1985; Oikari et al., 1985; Wood et al., 1989) and especially in laboratory studies with metals where disturbances to ionic regulation are a common feature of metal toxicity (Galvez and Wood, 2002; Grosell et al., 2004; Handy et al., 1999; Pane et al., 2003; Rogers et al., 2003).

Blood Metabolites and Enzyme Activities

Sample storage is particularly important issue for reliable measurement of these parameters. For field biologists, sample decay can be slowed by chilling samples and adding protease inhibitors. In laboratory studies, enzyme activities are generally measured in fresh blood samples or on samples that have been immediately stored at –80°C or under liquid nitrogen (Jayaram and Beamish, 1992). If chilled plasma or serum can be obtained, then determination of urea, glucose, amino acids, protein, immunoglobulins, and enzymes such as lactate dehydrogenase, acetylcholinesterase, and δ-aminolevulinic acid dehydratase (ALAD) is possible (Galloway and Handy, 2003; Haux et al., 1986; Jayaram and Beamish, 1992; Oikari et al., 1985; Sepúlveda et al., 2004). However, it is worth being selective in the context of the environmental contaminants of concern and existing knowledge on the biochemical effects of the pollutants. Here, a tiered approach is recommended (Galloway and Handy, 2003; Handy et al., 2003), where initial measurements may look for evidence of general stress, such as elevation of plasma glucose or lactate or increases in cortisol or adrenergic substances (McDonald and Milligan, 1997). Then, more specific indicators of particular groups of pollutants may be assessed—for example, ALAD for lead (Haux et al., 1986), esterase activity for organophosphate pesticides (Oikari et al., 1985; Thompson, 1999), or immune parameters for suspected immunotoxicants (Galloway and Handy, 2003).

Circulating Hormones

The endocrine responses that have traditionally been associated with stress (Brown, 1993; Sumpter, 1997) include: (1) the adrenocortical response involving cortisol secretion from the anterior region of the kidney; (2) the adrenergic response of catecholamine (adrenaline and noradrenaline) release from the chromaffin cells in the kidney; (3) prolactin secretion from the pituitary to produce compensatory changes in gill or skin permeability; and (4) thyroxine (T_4) secretion from the diffusely located thyroid follicles (teleosts do not have an anatomically distinct thyroid gland). There is also evidence that the adrenocortical and thyroid hormone responses are linked to the osmoregulatory role of growth hormone (Sakamoto et al., 1993). Insulin-like growth factor (IGF) has also been targeted in studies with nonylphenol (Arsenault et al., 2004). In addition to these responses, the effects of changes in the circulating levels of the sex hormones (testosterones or estrogens) or their mimics (estrogenic pollutants) have been the subject of much research (McMaster et al., 2001; Snyder et al., 2004; Sumpter 1997; Tyler et al., 1998).

Circulating levels of hormones are usually determined on chilled plasma samples, sometimes after storage at –20°C (Whitehead and Brown, 1989). The determination of cortisol, thyroxine, catecholamines, prolactin, and growth hormone can involve radioimmunoassays, sometimes with solvent extraction steps, or the use of combined radioenzymatic techniques (Goss and Wood, 1988; Harries et al., 1997; Pottinger et al., 1992; Sakamoto et al., 1993; Tyler et al., 1996; Whitehead and Brown, 1989; Witters et al., 1991). Similar to metabolites and enzymes as biomarkers, a tiered approach is recommended, starting with hormones associated with general stress responses (e.g., cortisol) (Benguira and Hontela, 2000; Sumpter, 1997), and then to specific hormones that may be associated with particular toxic effects (e.g., sex hormones for endocrine disruption or thyroid hormones for growth and development

effects) (Brown et al., 2004a; Eales and Brown 2005). Again, it is essential to have background information on the seasonal, species/strain, metabolic, and reproductive status effects, as well as the influence of general environmental conditions on blood hormone levels, so the effects of pollution can be separated from this background variability.

Condition Indices

Physical condition indices have been used for many years in aquaculture as a simple method for monitoring changes in fish health. These approaches index the physical dimensions of the animal or one of its internal organs (e.g., liver, spleen, gonads) with body weight. The most common morphometric index is the condition factor, expressed as weight (g)/length^3 (cm); however, organosomatic indices (ratios of organ mass to body mass) may provide more specific information sometimes relating to the function of the organ selected. Condition indices are also potentially very attractive biomarkers for field biologists because they are simple to perform, often requiring little more than a battery-operated balance and a ruler. The external measurements, such as the condition factor, are also of interest because they are nondestructive and noninvasive. Condition indices, like many other biomarkers, are influenced by environmental factors (e.g., season, temperature) (Imsland et al., 1995; Khallaf et al., 2003; Mustafa et al., 1991) and the physiological status of the animal (e.g., nutrition) (Russell et al., 1996). Thus, temporal changes in these indices may not be directly related to pollutant exposure. On the other hand, pollutants can produce rapid and marked changes in condition indices that are clearly differentiated from any underlying seasonal or life-cycle influence (e.g., in gonads) (Jobling et al., 1996). Goede and Barton (1990) reviewed condition indices and described how the indices may be scored as part of a quantitative autopsy technique, with obvious applications in aquaculture and aquatic toxicology. Attempts have also been made to correlate multiple sets of indices (e.g., weight, length, organ weights) to measure fish health at polluted and clean sites (see Table 16.6). This section briefly reviews the effects of pollutants on condition indices and highlights some of the compounding factors worth considering in field and laboratory studies. Some recent examples of the use of such indices in pollution studies are illustrated in Table 16.6.

Condition Factor

The condition factor relates body length to weight (weight/length^3). This measurement is common in fisheries and aquaculture because it is simple to perform; consequently, a growing body of literature relates the common environmental stresses in fisheries, such as stocking density (Wagner et al., 1997), dissolved oxygen (Miller et al., 1995), salinity (Craig et al., 1995), and food intake (Einen et al., 1998), to the condition factor. These stresses decrease the condition factor, which is often interpreted as a decline in body fat or stored glycogen in the liver. Similarly, pollutants that cause an increase in metabolic rate, a decline in energy intake, or enhance fat metabolism as part of the mode of toxic action, are likely to decrease condition factor (Smolders et al., 2003). Elevation of metabolic rate is a common response to pollutant exposure (Randall et al., 1996), and many pollutants cause a decline in energy intake either by changing feeding behavior (Little et al., 1985) or by toxic effects on absorption in the gastrointestinal tract (Clearwater et al., 2005; Handy et al., 2005). These factors in combination with lipid peroxidation of food items or the gut mucosa can cause a decline in condition factor.

Goede and Barton (1990) pointed out that, in wild fish populations, temperature, food availability, competition, and other factors may interact synergistically to produce a general decline in fish health. It may not be possible to elucidate pollutant effects on condition factor because the effects of pollutants on the condition factor are masked by other biotic or abiotic variables. Pollutants may therefore decrease the condition factor or have no apparent effect (see Table 16.6). In a study of the health and condition of the Pacific herring (*Clupea pallasi*) after the *Exxon Valdez* oil spill, Elston et al. (1997) concluded that the effects of reproductive stage, spawning behavior, and location were likely to invalidate interpretation of condition indices data. In a study of the metal contamination in the Clark Fork River in Montana (Farag et al., 1995), brown trout showed larger variation in the condition factor between reference sites (0.93 to 0.41) compared to that between contaminated sites (0.46 to 0.31); however, in

TABLE 16.6

Some Examples of Pollutant Effects on Condition Factors and Organosomatic Indices in Fish

Parameter and Species	Toxic Substance	Type of Response	Refs.
Condition factor			
Nile tilapia (*Oreochromis niloticus*)	Pesticides and metals in polluted canals in Egypt	Effects of pollution were greater than variation in condition factor associated with season or sex differences. Fish in the most polluted canal had the lowest condition factor (2.07), compared to 2.19–2.39 in other canals.	Khallaf et al. (2003)
Gudgeon (*Gobio gobio*)	River with discharge from a sewage treatment plant	Fish were larger at the reference site (both length and weight), but no site-dependent differences in condition factor were observed.	Faller et al. (2003)
Zebrafish (*Danio rerio*)	Sewage effluent from an industrial plant	After 4 weeks' exposure to effluent, condition factor was correlated with changes in energy budget and reflected changes in glycogen and lipid contents.	Smolders et al. (2003)
Lake whitefish (*Coreogonus clupeaformis*)	Chronic dietary exposure to nickel: 0–1000 mg Ni per g food for up to 104 days	No effect on growth or condition factor was observed.	Ptashynski et al. (2002)
Three-spined stickleback (*Gasterosteus aculeatus*)	Fish collected from 7 rivers classified as different quality by river ecosystem classes	Differences were noted in condition factors between sites within and between river catchment areas.	Handy et al. (2002b)
Fathead minnow (*Pimephales promelas*)	Domestic wastewater exposure for 3 weeks in field and laboratory conditions	Some wetland field sites showed a reduction in condition factor of fish, although it was not possible to identify the specific contaminants causing this effect.	Hemming et al. (2001)
Rainbow trout (*Oncorhynchus mykiss*)	Run off from copper mine tailings.	No differences in condition factor of fish between sites.	Dethloff et al. (2001)
Hepatosomatic index (HIS)			
Largemouth bass (*Micropterus salmoides floridanus*)	Paper-mill effluent exposure in the laboratory for up to 56 days and field collected animals	Effluent dilutions greater than 20% caused the HSI to rise after 56 days; for 20% and 80% dilutions, after only 28 days.	Sepúlveda et al. (2004)

Guppies (*Poecilia reticulata*)	Chronic sub-lethal nonylphenol exposure for 90 days	Newborn guppies, both males and females, showed an increase in the HSI during exposure compared to controls.	Cardinali et al. (2004)
Atlantic salmon (*Salmo salar*)	Dietary exposure to either 0–100 mg $HgCl_2$ or 0–10 mg MeHg per kg feed for 4 months	No effect on weight, length, or HIS was observed for either inorganic or methylmercury exposures.	Berntssen et al. (2004)
Fathead minnow (*Pimephales promelas*)	Domestic wastewater exposure for 3 weeks in field and laboratory conditions	Some wetland field sites showed a reduction in the HSI of fish; the gonadal somatic index (GSI) response was not the same as the HIS, as only one site showed a reduction in GSI.	Hemming et al. (2001)
Walleye (*Atizostedion vitreum*)	Walleye collected from mercury- and selenium-contaminated lakes	Liver selenium levels were inversely correlated with the HSI in both male and female fish.	Mauk and Brown (2001)
Gondal somatic index (GSI)			
Largemouth bass (*Micropterus salmoides floridanus*)	Paper-mill effluent exposure in the laboratory for up to 56 days and field collected animals	The GSI was higher in fish of both sexes from contaminated sites in the main stream, but not in the tributaries of the river system.	Sepúlveda et al. (2004)
Gudgeon (*Gobio gobio*)	River with discharge from a sewage treatment plant	No difference was observed in the GSIs between different sites, although the GSI was higher in females compared to males in the spring (seasonal effect).	Faller et al. (2003)
Zebrafish (*Danio rerio*)	Fish exposed to a range of concentrations of ethynylestradiol (EE2) or *p*-dichlorobenzene (DCB) for 14 days	The GSI showed more statistically different changes in females than in males; both EE2 and DCB tended to reduce the GSI. Vitellogenin levels in the plasma were correlated with GSI in females.	Versonnen et al. (2003)
Zebrafish (*Danio rerio*)	0–25 ng/L 17α-ethynylestradiol (EE2) exposure for up to 24 days	Females showed lower GSIs after 6-day exposures to 10 ng/L EE2 and after 24 days in males. Changes in the ovarian somatic index in female fish correlated with vitellogenin production.	Van den Belt et al. (2002)

the same study it was clear from tissue contaminant analysis and lipid peroxidation measurements that fish at the contaminated sites were suffering from toxic effects. It might therefore be preferable to choose a condition index that is more closely related to the target organ for the pollutant of concern. Alternatively, the condition factor may confirm the absence of toxic effects or indicate ecosystem recovery from pollution. In a study on the chronic toxicity of sediments containing drilling fluids, Payne et al. (1995) observed only minor changes in the condition indices of turbot which were supported by the absence of organ pathologies and dose effects in blood parameters. In a study of remediation in acidified lakes, Gloss et al. (1989) found an increase (recovery) in the condition factor of brook trout (*Salvelinus fontinalis*) after liming.

Organosomatic Indices

These indices measure organ mass relative to body mass (e.g., hepatosomatic index, liver weight/fish weight × 100%) and offer a big advantage over the condition factor as a biomarker in that they may relate directly to toxic effects on the target organ of contaminant exposure. Thus, the hepatosomatic index (HSI) might be an appropriate biomarker for substances that are toxic to the liver (e.g., cadmium) (Haux and Larsson, 1984), while the gonadal somatic index (GSI) may be more appropriate for estrogen mimics (Table 16.6) (Jobling et al., 1996). The hepatosomatic index is perhaps one of the most commonly applied indices because of the central role of the liver in the detoxification of pollutants (see examples in Table 16.6). Goede and Barton (1990), however, pointed out that the HSI may decline in response to starvation (glycogen depletion), but liver weight my increase due to pathological changes (e.g., hyperplasia associated with pollutant exposure). The resultant effect could, in theory, be no net change in HSI even if resultant histological examination of the tissue reveals toxic responses (Schwaiger et al., 1997; Teh et al., 1997); nonetheless, the HSI has proven useful in field and laboratory studies with fish (Table 16.6). Other organosomatic indices, including the splenosomatic index, viscerosomatic index (Goede and Barton, 1990), and relative ventricular weight (Armstrong and West, 1994), have been applied to studies on salinity, temperature, and seasonal change but remain to be validated as a technique for the toxicologist.

Growth

Growth rate has been used as an endpoint for many years in ecotoxicology. Measurements of growth can be simple measurements of body weight over time to calculate weight gain or mean daily specific growth rates (SGRs), or growth can be measured at the biochemical level in terms of changes in cell protein content (Smolders et al., 2003) or RNA/DNA ratios (Benton et al., 1994; Pottinger et al., 2002). The underlying assumption is that growth is reduced by pollutant exposure because energy intake has decreased or energy expenditure has risen because of the costs of avoiding exposure, detoxification, or tissue repair. The effects of contaminants on the growth of fish are inevitably measured during chronic sublethal exposure conditions, usually over several weeks rather than acute exposures of a few days. The effects of chronic dietary metal exposure on fish have recently been reviewed (Clearwater et al., 2005; Handy et al., 2005), and the growth rates of fish have also been measured during exposures to organic chemicals (Brown et al., 2002; Palm et al., 2003; Reiser et al., 2004). Clearly, growth rate is influenced by many factors, including food intake, the energy content of the food, water temperature, energy expenditure on swimming, and so on (Jobling, 1993). From the view point of biomarkers, we would like to differentiate these normal biological effects on growth from those of pollutants. This is relatively easy to do in the laboratory, and given sufficiently long enough time or high enough doses the growth rate can be impaired (e.g., for metals) (Clearwater et al., 2005). Many reports, however, have indicated that growth rates do not change very much during environmentally relevant exposures (Brown et al., 2002; Campbell et al., 2002; Handy et al., 1999; Palm et al., 2003; Reiser et al., 2004). This may be because the fish have altered their locomotion budget to save energy and preserve their growth rate during pollutant exposure (e.g., dietary copper) (Campbell et al., 2002; Handy et al., 1999), or, alternatively, the fish have made an endocrine adjustment that promotes growth. Enhanced metabolism of thyroid hormone (T_4), for example, may preserve growth rates during PCB exposure (Brown et al., 2004b). This is particularly interesting as it suggests that the hormones that control growth rate, rather than the growth rate itself, may be a potentially useful group of biomarkers in the future.

Respiratory and Cardiovascular Responses as Biomarkers

The main functions of the gills are in gas exchange (Randall and Daxboeck, 1984) and osmoregulation (Payan et al., 1984; Laurent and Hebibi, 1988). Investigations on respiration and circulation during exercise and hypoxia have revealed aspects of the control of gas exchange. Under normal physiological conditions, gas exchange is controlled mainly by adjustments in ventilation, while blood flow through the gills (perfusion) is kept reasonably consistent (Randall and Daxboeck, 1984). As the partial pressure of oxygen (pO_2) in the blood falls (e.g., during exercise or as a result of low environmental pO_2), then the ventilation rate increases and a marked fall in heart rate (bradycardia) is observed. Blood flow to the gills is maintained by an increase in cardiac stroke volume which offsets the bradycardia to maintain cardiac output for all but the most extreme cases of hypoxia in the blood of trout (Randall, 1982). Thus, the primary control of respiration appears to be efferent arterial pO_2 in fish. Changes in the partial pressure of carbon dioxide (pCO_2) which alter blood pH may influence pO_2 via acid–base effects on hemoglobin oxygen affinity and saturation (Eddy et al., 1977), so carbon dioxide can indirectly alter respiration, as well (Desforges et al., 2001, 2002; Perry and Gilmour, 2002). Adequate blood flow through the gills is also essential for normal physiological function; in fact, the ratio of water ventilation to blood perfusion is an important factor in maintaining optimum diffusion gradients across the gills. The ventilation/perfusion ratio is normally about 10 to 20 in fishes (Taylor, 1985). Blood flow is influenced by many factors (e.g., catecholamines, exercise), and fish tend to maintain blood flow to the gills by compensatory changes in heart function rather than direct changes in vascular resistance in the gills (Randall and Daxboeck, 1984). In addition to water and blood flow (diffusion gradients), gas diffusion and ion movements across the gills may depend on gill permeability, the functional surface area, and the diffusion distance between the water and blood. It is therefore not surprising that changes in the composition or status of the gill epithelial cells have profound effects on both respiration and osmoregulation (Bindon et al., 1994; Laurent and Hebibi, 1988). Thus, from a functional perspective any pollutant that ultimately alters blood pO_2, pCO_2, pH, vascular resistance, or blood flow (heart function) or induces changes in gill cell proliferation will probably cause a change in respiratory function.

Laboratory studies have shown that acute exposure to aquatic pollutants can cause edema and epithelial lifting in the gills (Alazemi et al., 1996; Mallat, 1985; Skidmore and Tovell, 1972), which may be accompanied by the hypersecretion of mucus (Handy and Eddy, 1989). These structural effects will at least increase the diffusion distance for the respiratory gases to cause hypoxia or hypercapnia (Piiper, 1998; Sellers et al., 1975), which ultimately leads to changes in ventilation frequency and volume (Sellers et al., 1975). Pollutants that deoxygenate or acidify the water may also stimulate ventilation (Wright et al., 1986) in the absence of gross gill pathology. Chronic sublethal exposure to pollutants may have more subtle effects on respiratory and cardiovascular responses. Fish may show anatomical adjustment of gill surface area in the form of changes in the length or thickness of the gill filaments (Handy et al., 1999; Wilson et al., 1994) or altered proportions of the cell types in the epithelia (Wendelaar Bonga et al., 1990), which may result in the need for only minor changes in ventilation in the long term to retain physiological function. Alternatively, fish may reduce energy expenditure on activities such as locomotion in order to minimize extra demands on respiration during pollutant exposure (e.g., dietary copper) (Campbell et al., 2002; Handy et al., 1999).

The question arises as to which of these responses can be used as a biomarker and which parameters (blood gases, oxygen uptake, ventilation frequency and volume, blood flow, blood pressure, cardiac output, or its components) can be measured on a routine basis so the biomarker can be used in environmental monitoring programs as well as fundamental research (Handy and Depledge, 1999; Handy et al., 2002a). In the laboratory, evaluating blood gases and blood pressure and conducting electrocardiograms usually require invasive techniques (Houston, 1990), although non-invasive approaches have also been tried for ventilation (Kramer and Botterweg, 1991). Optical techniques such as online cardiovascular monitoring have been developed for crustaceans, but these have not been tried on fish species (Handy and Depledge, 1999). Oxygen consumption rates measured in a fish respirometer are particularly useful, as this measurement indicates the aerobic cost of pollutant exposure (Campbell et al., 2002). For aqueous exposures, at least, correlations exist between oxygen consumption rates and xenobiotic transfer rates across the gills (Randall et al., 1996). Oxygen consumption may therefore be a useful tool for predicting branchial exposure, and several models have been suggested (Yang et al., 2000a,b). Whatever

biomarker of respiratory function is selected, it is important to measure the variability of response in addition to absolute values, so a normal range can be established (Handy and Depledge, 1999). Only then will it be statistically possible to separate normal biological variation such as diurnal changes in metabolism, food intake, water quality, and temperature (Gonzalez and McDonald, 1992; Lyndon et al., 1992; Morgan and Kühn, 1984; Wilson et al., 1994) from the effects of pollution.

Behavior as a Biomarker

A variety of animal behaviors can be measured during pollutant exposure, including avoidance of the pollution gradient, changes in feeding activity, predator avoidance, foraging behavior, reproductive behavior, social behavior, and swimming behavior (Kasumyan, 2001; Little and Finger, 1990; Little et al., 1985; Sandheinrich and Atchison, 1990; Schreck, 1990; Scott and Sloman, 2004). Behavioral biomarkers offer three very important advantages over biochemical, morphological, or physiological biomarkers: (1) The behavioral response is often an integrated effect of the underlying biochemical and physiological disturbances and so may reflect a series of toxic effects and compensatory responses (Campbell et al., 2005). (2) Behavioral responses are often more sensitive indicators of exposure than other approaches (Little and Finger, 1990). (3) Some behavioral responses can be linked on an energetic basis to population survival (e.g., locomotor activity) (Handy et al., 1999; Priede, 1977).

Ecotoxicological research on fish behavior in the laboratory has studied both metals and organic pollutants (Little et al., 1985; Scott and Sloman, 2004) but has generally focused on relatively short-term acute effects lasting a few hours or days (Little and Finger, 1990; Rice et al., 1997; Scarfe et al., 1982). Most studies have focused on exposure via the water, where disturbances to respiration and osmoregulation (Pilgaard et al., 1994) may limit locomotor capacity (Waiwood and Beamish, 1978) and thus reduce the behavioral repertoire of the animal. In addition, alterations of olfaction by various organic and inorganic contaminants may also significantly impact grouping and schooling behavior, thus allowing animals to be more susceptible to predation (Sandahl et al., 2005; Scholz et al., 2000). Alternatively, food route exposures may not damage the gills, so the behavioral activities of the fish are not limited by respiratory distress (Handy et al., 1999). Furthermore, the etiology of brain injury may be different in dietary compared to aqueous exposures (e.g., mercury) (Berntssen et al., 2003). We should therefore consider that behaviors not only have the opportunity to increase in complexity over time in chronic compared to acute exposures (Scott and Sloman, 2004) but may also vary with the route of exposure. Some behaviors may also be attenuated, such as the loss of circadian rhythms and aggression during dietary copper exposures (Campbell et al., 2002, 2005). This raises the possibility of biomarkers based on the presence or absence of behavioral responses.

Sudden changes in fish behavior, such as altered swimming ability, have long been suggested as early warning systems or as biological monitoring tools for acute pollution. Early biomonitoring devices required animals to be housed in a flow-through system with the recording apparatus in close proximity. Today, acoustic tags, radio tags and transponders, and cardiovascular monitoring devices have enabled wildlife telemetry to be employed in more realistic field situations on unrestrained fish (for a discussion of these technologies in biomonitoring, see Handy et al., 2002a, and references therein). Such biomonitoring technologies for measuring fish behavior could be applied to the biomarker concept. Similar to biomonitoring, any device used to assess behavior as a biomarker must be robust, reliable, and validated against a variety of manual or other techniques for observing behaviors (Craig and Laming, 2004). Systems such as the Multispecies Freshwater Biomonitor have been used to assess locomotor behaviors in the presence of environmental stressors such as ammonia (Craig and Laming, 2004) or municipal wastewater (Gerhardt et al., 2002), and similar systems have been used for fish or invertebrates with a variety of pollutants (Handy and Depledge, 1999; Handy et al., 2002a); however, it remains a challenge to use these techniques as biomarkers of chronic pollution. Most of the existing data on behavioral effects are over time scales of a few hours or days, although Scott and Sloman (2004) pointed out that experiments on complex behaviors such as reproductive behaviors and some social behaviors involve longer time scales. We are also now realizing that changes in behavior feed back at the physiological level to produce adaptive changes in physiology (Scott and Sloman, 2004); for example, during metal exposure such changes include changes in the cost of aerobic metabolism following the loss of circadian

rhythms (Campbell et al., 2002) and adjustment of ion flux rates with social status (Sloman et al., 2002). Behavioral biomarkers may therefore be usefully applied and integrated with other physiological measurements to give an overall picture of biological effect at the whole organism level.

Pathological Biomarkers

Sublethal exposure to environmental chemicals may result in changes in the histological structure of cells and the occurrence of pathologies which can significantly modify the function of tissues and organs. Use of histopathological techniques in a biomarker approach has the advantage of allowing investigators the opportunity to examine specific target organs and cells as they are affected by exposure to environmental chemicals. Additionally, histopathology provides a means of detecting both acute and chronic adverse effects of exposure in the tissues and organs of individual organisms. Cells are the component units of organization in each tissue, and they show a variety of structural and functional specializations, based on the quantitative differences in individual organelles. Cellular organelles may be considered highly conserved structures, as they are recognizable in finfish as well as shellfish. Hinton (1994) suggested it is the conservative nature or common features of cellular organization in vertebrate and invertebrate organisms that make histopathological examination a valuable tool in the biomarker approach. Use of molecular responses within aquatic organisms in the biomarker approach has been reviewed, and it was noted that such responses occur early and are typically the first detectable quantifiable response to exposure to environmental chemicals. Linkage of molecular events to damage at the cell and higher levels of biological organization is only beginning to emerge, and greater effort is being directed toward determining the significance of molecular events to subsequent forms of cellular injury and response (Au, 2004; Myers and Fournie, 2002). Hinton et al. (1992) suggested the integration of histopathology with biochemical and physiological biomarker approaches, as histopathological alterations are the net result of adverse biochemical and physiological changes in an organism. Because genes control cell products and therefore appearance of specific cells, it is apparent that a focus on cells would enhance the ability to detect common mechanisms of action. Histopathological biomarkers are considered higher level biological responses and often signify prior metabolism and macromolecular binding. Most chemicals that are potentially genotoxic require metabolic activation to an ultimate form that binds covalently, forming adducts to DNA. If the adduct is not repaired and persists, subsequent changes lead to a multistep process that could result in cell death or perhaps abnormal growth and tumor formation (Figure 16.6). In the latter case, the histopathologic biomarker is a higher level response following chemical and cellular interaction. Similarly, exposure to an environmental chemical may induce the activity of a specific enzyme or enzyme isoform. Subsequent exposure could lead to increased metabolism by the induced enzyme, resulting in levels of toxic intermediates that exceed cellular detoxification mechanisms; therefore, induction and metabolism could lead to cellular toxicity and death, subsequently detected as tissue necrosis or apoptosis.

Morphological Abnormalities

The view that disturbances in structure or function of individual cells form the basis of a disease state or, in this case, toxicity was first put forward in the 19th century with the concept of "cellular pathology" suggested by Rudolf Virchow, known as the father of pathology (Cotran et al., 1994). Pathology has been an indispensable and robust technique in established routine toxicology studies in rodents, performed for the purpose of risk assessment. Its value lies not only in the sensitivity with respect to establishing thresholds of toxicity but particularly in the identification of target organs and mechanisms of action. In aquatic toxicology, the use of the histopathologic approaches in evaluating toxicologic pathology of organisms has been shown to have a number of strengths.

The documentation of neoplasms in aquatic organisms was perhaps the first use of histopathological indices in the biomarker approach to environmental monitoring and began as early as the 1800s with the description of tumors in fish and mollusks. By the 1960s, several epizootic neoplasms had been reported in feral fish. The connection between epizootic neoplasms in feral fish and the chemical

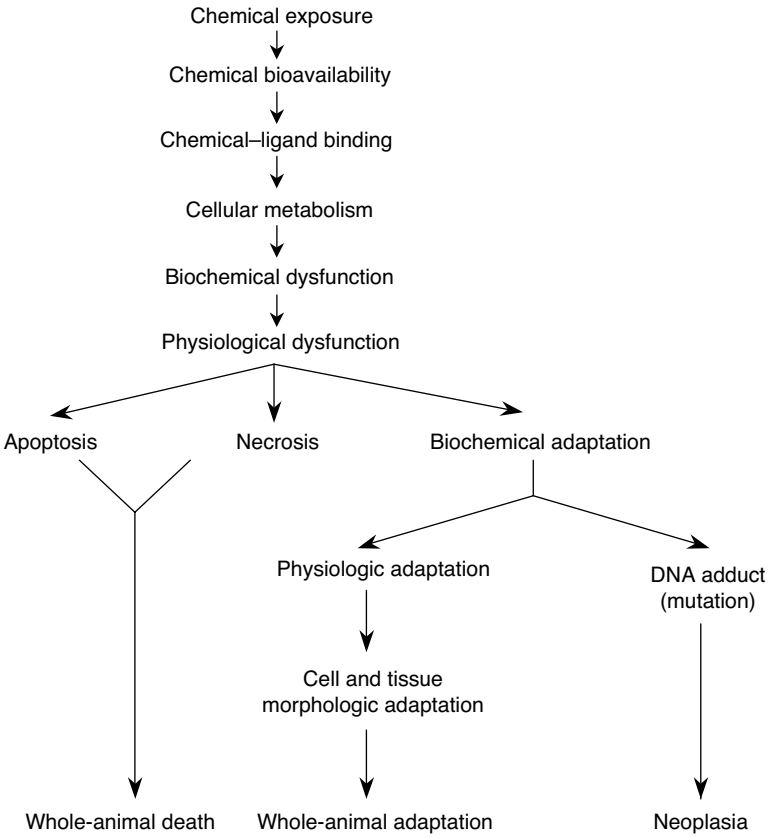

FIGURE 16.6 Cascade of histopathological alterations as result of biochemical and physiological alterations in an organism. (Adapted from Hinton, D.E. and Laurén, D.J., in *Biological Indicators of Stress in Fish*, Vol. VIII, Adams, S.M., Ed., American Fisheries Society, Bethesda, MD, 1990, pp. 51–66; Hinton, D.E. et al., in *Biomarkers: Biochemical*, *Physiological*, *and Histological Markers of Anthropogenic Stress*, Huggett, R.J. et al., Eds., Lewis Publishers, Boca Raton, FL, 1992, pp. 155–209.)

contamination of aquatic ecosystems was first suggested by Dawe et al. (1964), who postulated that liver neoplasms observed in white sucker and brown bullhead from Deep Creek Lake in Maryland might have resulted from chemical exposure. Since that time, numerous wild fish tumor epizootics have been identified in North America alone, dominated by liver tumors in bottom feeders in the vicinity where chemical contaminants were concentrated. Pierce et al. (1978), for example, reported the occurrence of hepatic tumors in English sole in Puget Sound in Washington. Malins et al. (1984) also studied sediment-associated chemicals from near-coastal areas and related PAHs to the induction of tumors in marine fish species. A good correlation, in fact, was found between the indices of hepatocellular carcinoma or papilloma of the fish examined and PAH sediment concentration. Likewise, Black (1983) reported that a high prevalence of epidermal papilloma, epidermal carcinoma, and hepatocellular carcinoma was observed in brown bullhead inhabiting the Buffalo River, in which sediment was contaminated with PAHs. Harshbarger et al. (1993), and Moore and Myers (1994) provided additional fish histology references focused on the pathobiology of chemical-associated neoplasia in aquatic organisms.

Morphological change can manifest itself in various external and internal lesions or diseases that can be easily quantified (Au, 2004). External metrics include fin erosion, skeletal abnormalities, epidermal hyperplasias, and opercular abnormalities (Au, 2004). In addition, a number of characteristics of anatomical and cytological alterations favor the use of histological examination in the biomarker approach (Myers and Fournie, 2002). With a thorough prior knowledge of normal anatomy, the use of histological analysis can be used to detect chemically induced alterations in a variety of tissues and organs in many

different aquatic organisms. However, as with the use of other biomarkers, histological alterations may be influenced by factors other than chemical exposure. Given age, diet, environmental factors, seasonal variation, and reproductive cycle, several structural states may represent normality and could be potentially confounding issues in an attempt to use histological criteria as biomarkers. Regarding specific alterations and references on histology of aquatic organisms, the reader is referred to Ashley (1975), Au (2004), Couch and Fournie (1993), Ferguson (1989), Grizzle and Rogers (1976), Hinton et al. (1992), Kubota et al. (1982), Myers and Fournie (2002), and Yasutake and Wales (1983).

Toxic effects on biochemical and physiological systems are ultimately expressed as changes in cellular and subcellular morphology; thus, histopathological changes may be viewed as an integration of molecular insults (Hinton and Lauren, 1990). Depending on the size of the fish, a number of important tissues may be simultaneously studied while maintaining *in situ* cellular, tissue, and organ system relationships. Maintenance of spatial relationships is possible and, in fact, required to appreciate biological effects associated with toxicity in localized portions of an organ and the subsequent derangements in fluids, tissues, or cells at other locations (Hinton, 1993). With small adults or fish larvae, proper orientation for hemisection may yield samples through major viscera that can be viewed on only a few histological slides. Such an approach enables examination of many potential targets of toxicant action and has been used very effectively by a number of investigators. In medaka (*Oryzias latipes*) exposed to the estromimetic chemical hexachlorocyclohexane, Wester and Canton (1986) evaluated morphological changes in gonads, liver, kidney, pituitary, thyroid, spleen, and heart. The organs, or portions of organs, affected are recognized targets of estrogen or are involved in the metabolism of products resulting from estrogenic responses.

Histopathologic analysis has proven to be a useful component in biomarker approaches to evaluate the potential risk to aquatic organisms posed by exposure to both natural and anthropogenic chemicals that may interfere with reproduction and development. In most fish, sex determination is under genetic control; sex is determined by sex chromosomes after fertilization. In some fish, autosomes (pairs of chromosomes with sex modifying genes) determine the sex, but this is less common (Jobling, 1995). Sexual differentiation in fish is believed to be similar to mammalian systems whereby the presence or absence of a testis-determining factor directs male or female differentiation. In teleosts, primordial germ cells develop exterior to the gonadal region and then undergo migration to the gonad. The development and positioning of the primordial germ cells are important markers of sexual differentiation, which is a species- as well as sex-dependent process. In most cases, the differentiation of the gonad occurs in females before males. This is usually determined by enumerating the number of primordial germ cells entering or undergoing meiosis; for example, sexual differentiation in Japanese medaka occurs at hatching, and in female medaka germ cells are in a proliferative state. Kanamori et al. (1985) reported that sexual differences in the medaka were identifiable in the male medaka 10 days after fertilization. The actual tubular structure of the testis was not seen until the fish was 15 to 20 mm in length, which corresponds to the time when meiosis should occur (Kanamori et al., 1985). In some species, sexual differentiation can be marked by the presence of an ovarian or testicular cavity. The evaluation of gonadal development has been used as a biomarker response in field studies that have evaluated sexual determination and differentiation. Jobling et al. (1998), for example, demonstrated a high incidence of intersexuality in populations of roach (*Rutilus rutilus*) in the United Kingdom. The intersex condition was characterized by the appearance of female characteristics in a typically male tissue and the progressive disappearance of male characteristics. The investigators reported that the reproductive disturbances were consistent with exposure to hormonally active substances and were associated with discharges from sewage treatment works known to contain estromimetic chemicals.

For decades following the introduction of the concept of cellular pathology by Virchow, the understanding of morphological abnormalities at the cellular level was limited to data derived from fixed images of cells and tissues as seen through the light microscope. Although the subjective nature of conventional histopathology did not detract significantly from the discriminating power of this approach, recent technological advances have improved resolution and identification of specific cellular and macromolecular markers. Morphological changes in lysosomes, for example, have been employed as a biomarker to evaluate toxic liver injury in various fish species (Kohler et al., 2002). Additionally, technological advances in the field of histopathology have enabled increased quantitative assessment using techniques such as digital optical imaging and immunohistochemical techniques.

Immunohistochemistry

Immunohistochemistry of routinely fixed and processed tissues has been one of the major technological advances in histological evaluation and diagnosis, because it represents an independent, objective method of cell identification against which traditional subjective morphological criteria may be compared. Since its inception by Coons et al. (1941), immunohistochemistry has been used in many aspects of investigation of biological materials. Its application has been particularly useful in the characterization of human neoplasms and in traditional mammalian toxicology. Although not widely employed in routine histopathological evaluation, immunohistochemistry represents a dynamic technique for addressing certain problems in aquatic toxicology. Many of the commercially available monoclonal and polyclonal antisera cross-react well with the tissue of aquatic organisms and can be used in the histological evaluation of toxicity studies, provided appropriate controls are applied. Examples include proliferating cell nuclear antigen (Ortego et al., 1994, l995) and the 170-kDa plasma membrane protein P-glycoprotein (Hemmer et al., 1998). When evaluating the induction of CYP1A proteins, the use of immunohistochemistry has proven to be very useful in the analysis of embryos or an organ for which other biochemical assays would be very difficult. Analysis of induction by immunohistochemical assays has further advantages in that it can reveal the cell types where induction occurs. The rationale for evaluating the activity of enzymes in the focal lesions is their involvement in the metabolic activation and inactivations of toxicants, with decreases in phase I and increases in phase II enzymes serving as an adaptive response to exposure to environmental chemicals. Several investigators have utilized immunohistochemical techniques to study cytochrome P4501A in scup (Miller et al., 1988, 1989; Stegeman et al., 1991), rainbow trout (Miller et al., 1988, 1989), and winter flounder (Smolowitz et al., 1989). Expression of other enzyme systems such as glutathione *S*-transferase (GST) have been examined immunohistochemically in rainbow trout (Kirby et al., 1990a), suckers (Kirby et al., l990b), and mummichog (Van Veld et al., 1991). Because of the possibility that changes in one or several enzymes may precede the development of altered foci, their utility as early morphologic indicators of chemical exposure and effect deserves further exploration.

Summary

This chapter attempted to systematically present a portion of the better characterized biomarkers with respect to their description and functional use. It is recognized that the biomarker approach in environmental monitoring is beneficial but must be viewed with respect to evaluation of exposure, effect, and susceptibility. Each of these categories can provide valuable data in the conduct of ecological risk assessments. The development and use of biomarkers have been, for the most part, at the molecular or cellular level of biological organization, but biomarker strategies must be utilized in an integrated approach in which a hierarchy of responses is evaluated. This hierarchy can be constructed based on the levels of biological organization that are monitored or on different degrees of response sensitivity.

The description and functional uses of biomarkers have been presented via a tiered evaluation of three systems focusing on biochemical, physiological, and pathological responses. An assessment of these systems is critical to understanding the basic mechanisms of fundamental toxicological processes. Biochemical alterations are typically considered the initial response to toxic insult by a xenobiotic. The biochemical biomarkers discussed included inducible proteins such as CYP1A, metallothioneins, heat shock proteins, and P-glycoprotein. Phase II biotransformation and antioxidant enzymes as well as various metabolites, including FACs, porphyrins, and retinoids, were also presented (in addition to genotoxic responses). Measurement of these biochemical endpoints can be extremely sensitive indicators of altered cellular function; however, the interpretation of biochemical responses as biomarkers should be evaluated carefully and with an understanding of the normal homoeostatic roles for these mechanisms. In addition, recent advances in -omics-based technologies present an opportunity to contribute significantly to improvements in determining the mode of action of environmental contaminants and in the further development of biomarkers. As an example, the development of gene, protein, or metabolite profiles could allow for more accurate characterization of the mode of action of a chemical. The development of such -omics-based profiles will contribute to our understanding of the variability of

-omic responses and, in turn, assist in distinguishing compensatory and adaptive responses from toxicologically significant responses. As the -omic technologies themselves do not distinguish compensatory and adaptive responses from causative events, the potential involvement of any gene, protein, or metabolite must be established by correlating the observed changes in -omic responses with physiological and pathological endpoints.

Hematology and clinical chemistry as well as condition indices were discussed as physiological biomarkers. Because the occurrence of pathologies can significantly modify the function of tissues and organs, an evaluation of pathological biomarkers was presented. Use of histopathological techniques permits the examination of specific target organs and cells as they are affected by either acute or chronic effects of xenobiotics.

This chapter emphasized the significance of biochemical, physiological, and pathological responses measured in individual organisms. These responses provide information concerning the exposure, effects, and susceptibility of aquatic organisms in the context of environmental monitoring and the biomarker approach. Much of the focus in aquatic toxicology is the elucidation of effects at biological levels of organization higher than that of the individual. Nonetheless, there exists considerable value in the development, implementation, and use of biomarkers due to the need for mechanistic evaluation of environmental chemicals and in recognition of the role that biomarkers can play in ecological risk assessment. It is hoped that with the advances made in proteomics and array technologies mechanism-based responses can be identified that will enhance predictive capacity and diminish uncertainty in risk evaluations.

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17

Aquatic Ecosystems for Ecotoxicological Research: Considerations in Design Analysis for Fish

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CONTENTS

Introduction

Environmental managers responsible for assessing the ecological integrity of aquatic resources rely on a number of assessment tools including chemical analyses of water, sediment, and fish tissue; biological assessments; and toxicity tests. Toxicity testing, under both field and laboratory conditions, is important for assessing chemical impact on aquatic ecosystems. In aquatic toxicity tests, groups of selected organisms are exposed to test materials (water or sediment samples) under defined conditions to determine potential adverse effects. Detailed guidance manuals for marine and freshwater toxicity tests are available from the U.S. Environmental Protection Agency (USEPA) and other nongovernmental groups such as the American Society for Testing and Materials (ASTM) or the American Public Health Association

(APHA). Consensus protocols provide guidance on the application of toxicity tests for assessing the toxicity of single chemicals, complex effluents, and ambient samples of water or sediment. Many fish species are used in laboratory toxicity tests (see Chapter 15 in this text). Such tests typically use one species, and have a duration of 96 hours or longer (APHA, 1999). Embryo–larval development tests and a 7-day larval growth and survival test have been developed using *Pimephales promelas* to represent freshwater fish; marine fish are represented by the sheepshead minnow (*Cyprinodon variegatus*), a species indigenous to the Atlantic and Gulf coasts (USEPA, 1994).

Several research studies have used model aquatic ecosystems of varying design and complexity to evaluate contaminant fate and effects in aquatic ecosystems. Such systems are designed to simulate ecosystems or portions thereof. As research tools for fish toxicity testing, model ecosystems contribute to our understanding of the manner in which contaminants affect fish populations in aquatic ecosystems (Crossland et al., 1993). If designed well, these systems allow ecologists to address hypotheses on a manageable scale and with control or reference systems. They also provide ecotoxicologists with models of ecosystem functioning in the absence of perturbation so direct and indirect effects might be better separated from natural events such as succession or inherent variation (Crow and Taub, 1979). Traditionally, model ecosystems are referred to as either *microcosms* or *mesocosms*. The distinction between microcosm and mesocosm is subjective and is mainly a function of size or volume (Giesy and Allred, 1985). Degrees of organizational complexity and realism often vary in these systems, depending largely on study goals and endpoints.

Giesy and Odum (1980) defined microcosms as "replicable, artificially bounded subsets of naturally occurring environments." Microcosms can contain several trophic levels and exhibit system-level properties. Mesocosms are defined as either physical enclosures of a portion of a natural ecosystem or manmade structures such as constructed ponds or stream channels (Voshell, 1990). Mesocosm size and complexity should be of sufficient size for the system to be self-sustaining and hence suitable for long-term studies of fish growth and reproduction (Voshell, 1990). In this regard, they differ from microcosms, where smaller size and fewer trophic levels do not allow for long study durations, particularly with fish. Cairns (1988), however, did not distinguish between microcosms and mesocosms because "both encompass higher levels of biological organization and have high degrees of environmental realism." Lack of a defined distinction between microcosms and mesocosms has caused some confusion among researchers around the world. The European Workshop on Freshwater Field Tests operationally defines microcosms on the basis of size, defining outdoor lentic microcosms as surrogate ecosystems of volume ≤ 15 m^3 and mesocosms as ponds of 15 m^3 or larger. Experimental stream channels were also characterized on the basis of size. Microcosms are defined as smaller and mesocosms larger than 15 m in length. In this chapter, we define model systems based on the European Workshop on Freshwater Field Testing (EWOFFT) definitions.

Mesocosms as Model Ecosystems

The use of model systems in aquatic research has grown considerably since the use of replicated ponds in community structure analysis in the late 1960s by Hall et al. (1970) and the pesticide studies of Hurlbert et al. (1970). Aspects such as community composition and spatial heterogeneity can be controlled to a greater extent in model (constructed) systems relative to natural ones. Model ecosystems are logistically more manageable and replicable for statistical analyses. In addition, model systems are effective tools in aquatic research because they allow a focus on important cause-and-effect pathways expected to occur in natural systems (Cairns, 1988; Odum, 1984). For fish, model ecosystems can retain a high degree of environmental realism relative to laboratory single-species toxicity bioassays (Cairns, 1988). Single-species tests are inadequate when chemical fate is altered significantly under field conditions, when organismal behavior can affect responses to a toxicant, or when indirect effects occur due to alterations in competitive or predator-prey relationships (La Point et al., 1989). Mesocosm tests, however, should be viewed as part of a tiered testing sequence and not as replacements of single-species bioassays (Cairns, 1989). The ability to detect and accurately measure responses of fish populations in mesocosms can be influenced by system characteristics and experimental designs that influence variability. If fish growth and reproduction are to be endpoints of interest in mesocosm testing, all key

environmental factors that influence fish growth, *independently of any chemical dosing*, must be understood. Chemical dosing can be viewed as a probe with which to perturb the system. Quantifying biotic responses to such perturbations helps us understand basic fish population dynamics.

Historical Perspective

Forbes (1887), in his work on lake natural history, detailed basic principles of ecological synergism, variability, and dynamic equilibrium, as well as the complex interactions of predator and prey. Though speaking of the lake itself, not of the surrogate systems routinely employed in aquatic research today, Forbes touched on the rationale for using artificial systems in both toxicological and ecological research: "It forms a little world within itself—a microcosm within which all the elemental forces are at work and the play of life goes on in full, but on so small a scale as to bring it easily within the mental grasp." This postulates the underlying basis for using microcosms (and mesocosms) in ecotoxicological research: the assertion that they simulate processes that occur in nature enough to be viable surrogates for natural systems.

Initial applications of artificial aquatic systems such as laboratory microcosms, artificial ponds, and various *in situ* enclosures were historically used in ecological studies of productivity (Kevern and Ball 1965; McConnell, 1965), community metabolism (Beyers, 1962, 1963; Copeland, 1965), and population dynamics (Deegan et al., 1997; Stein et al., 1995; Vanni et al., 1997). This research helped lay the groundwork for understanding how biotic processes function in artificially bounded and maintained systems. A fundamental knowledge of systems ecology is necessary if there is to be any understanding of how introduced perturbations (e.g., chemical insult) may be measured over and above natural perturbations of competition, predation, or background chemical and physical milieu. A subject of considerable concern and debate is whether microcosms simulate natural systems closely enough to be used as ecosystem surrogates, particularly when fish are included. Microcosms typically do not closely simulate natural systems at all levels of ecological organization. The small scale of microcosms has not been a problem for plankton or invertebrates, but their use remains problematic for fish.

The use of surrogate systems in toxicological research, particularly those encompassing any appreciable scale or complexity, is relatively recent (ca. 1960). Concern over the effects of insecticides used to control mosquito populations in California prompted a series of field studies on the consequences of chemical control methods on non-target species such as mosquito fish (*Gambusia* sp.) and waterfowl. Keith and Mulla (1966) and Mulla et al. (1966) used replicated artificial outdoor ponds to examine the effects of organophosphate-based larvicides on mallard ducks. Hurlbert et al. (1970) conducted subsequent studies in the same systems, examining the impact of this class of chemicals on a greater number of species within several broad taxa: phytoplankton, zooplankton, aquatic insects, fish, and waterfowl. Essentially, system-level responses attributable to the pesticide were studied with concomitant changes in the fish or waterfowl population of interest.

Broad application of microcosms and mesocosms in toxicological studies arose after the realization that single-species toxicity tests, alone, were inadequate for predicting effects at the population and ecosystem levels (Cairns, 1983; Kimball and Levin, 1985). It was felt that single-species laboratory toxicity tests were protective, but not predictive, of ecosystem responses (Fairchild et al., 1992). In addition, multispecies tests can demonstrate effects not evident in laboratory tests that use a single species (Cairns, 1986). As environmental protection goals focus on ecosystem-level organization, testing with more complex systems may involve less extrapolation, presumably with an enhanced capability of predicting impacts on natural systems. Yet, it remains a fact that the optimal use to which stream mesocosms have been put is to help understand the complexity of factors influencing growth and survivorship (Brooks et al., 2004; Stanley et al., 2005). In those studies, fathead minnows (*Pimephales promelas*) and amphipods (*Hyalella azteca*) were used as focal species to learn how aqueous cadmium moves through the food chain in model ecosystems.

The ecological risk of pesticide application is assessed under the U.S. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). The data collection process detailed under FIFRA involves a progression of increasingly complex toxicity tests, considered together with an estimate of environmental exposure, to make a determination whether a chemical may pose an unacceptable risk to the aquatic environment.

Much of this testing is focused on aquatic invertebrates and fish. Increasingly more complex tests are conducted in each tier, moving from simple acute tests to chronic to full life cycle. At each tier, data are evaluated and an estimate of potential risk to the aquatic environment is determined. Based on the outcomes of testing at each tier, the decision is made whether to stop testing or continue to the next tier. The final tier (Tier IV) involves field testing (AEDG, 1992). Registrants may be required by the EPA to conduct higher tiered tests or may voluntarily opt for this level of testing to refute the presumption of unacceptable environmental risk indicated by a lower tiered test. Generally, the fourth tier of testing has been viewed as tests to demonstrate that the chemical exposure, under more realistic environmental conditions, may not be as severe as expected under laboratory "clean-water" exposures. In effect, the fourth tier looks at how the chemicals dissipate, metabolize, hydrolyze, photolyze, and disperse and to where they ultimately move.

Prior to the adoption by the EPA of the mesocosm testing as part of the ecological risk assessment of pesticides, Tier IV tests were conducted in natural systems exposed to the agricultural chemical during the course of typical farming practices. Whereas these types of studies provided realism in terms of environmental fate of the compound and exposure to the aquatic ecosystem, they were difficult to evaluate, in part, because of insufficient or no replication, a high degree of variability associated with the factors being measured and influences of uncontrollable events such as weather. In the mid-1980s, the EPA adopted the use of mesocosms (experimental ponds) as surrogate natural systems in which ecosystem-level effects of pesticides could be measured (Tier IV tests) and included in the ecological risk assessment process (Touart, 1988). Although no longer part of the regulatory requirements in the United States, mesocosm tests requirements have stimulated an increased worldwide interest in the use of surrogate ecosystems for the evaluation of the fate and effects of contaminants in aquatic ecosystems as evidenced by the number of symposia and guidelines (Campbell et al., 1999; Giddings et al., 2002; Graney et al., 1994; Hill, et al., 1994a; SETAC–Europe, 1991; SETAC and RESOLVE, 1991).

Biomagnification

One of the best uses of enclosures or mesocosms for fish studies is to learn aspects of contaminant bioaccumulation into top predators. Barron (1995) presented an overview of biomagnification principles and determinants in aquatic food webs. Biomagnification is the increase in contaminant body burden caused by contaminant transfer from lower to higher trophic levels (Thomann et al., 1992). Rasmussen et al. (1990) showed that polychlorinated biphenyls (PCBs) in lake trout (*Salvelinus namaycush*) increased with length of the benthic-based food web and with tissue lipid content. Bioaccumulation into other keystone species, not necessarily just fish, has also been studied. Simon et al. (2000) analyzed the trophic transfer of cadmium and methylmercury between the Asiatic clam (*Corbicula fluminea*) and crayfish (*Astacus astacus*). Their results suggest a small risk of cadmium transfer between crayfish and predators, including fish and humans; however, methylmercury distribution in muscle and its consequent bioaccumulation in predators present an obvious risk.

Model Ecosystems

A wide variety of model ecosystems have been developed and used for fundamental and applied aquatic ecological research. Review articles describing these systems are available for microcosms (Giddings, 1980), freshwater mesocosms (Hill et al., 1994a; Solomon and Liber, 1988), marine mesocosms (Gearing, 1989; Grice, 1982; Lalli, 1990), and artificial streams (Kennedy et al., 1995).

Microcosms

Microcosms have been employed extensively in studies of contaminant effects on community-level structure and function. These systems can be viewed as an intermediate to laboratory tests and larger scale mesocosms. Microcosms, whether indoor or outdoor, may not accurately parallel natural systems at all levels of organization, but important processes such as primary productivity and community

metabolism can be studied in them, even in cases where systems cannot support all of the trophic levels found in larger systems. Outdoor microcosms have taken a variety of forms, including small enclosures in larger ponds (Maund et al., 1992; Schuaerte, 1982; Yasuno et al., 1988; Zrum et al., 2000) and freestanding tanks of sizes ranging from small (12-L) aquaria suspended in a natural pond (Lay et al., 1987) to vessels constructed of fiberglass (Drenner et al., 1987; Giddings et al., 1996; Howick et al., 1994; Kennedy et al., 1991; Rand et al., 2000; Shaw and Manning, 1996), stainless steel (Heimbach 1992), or concrete (Hill et al., 1994a; Johnson et al., 1994; Kedwards et al., 1999b), or excavated from the earth (Heimbach et al., 1992; Lucassen and Leewangh, 1994). Other researchers have used plastic wading pools (Scott and Kaushik, 2000) or temporary pond microcosms (Barry and Logan, 1998). Likewise, an assortment of mesocosm ecosystems has been devised. Mesocosms can be categorized into types or styles, based on their construction and size.

Enclosures

Canadian researchers have used limnocorrals, large enclosure systems in open-water areas of lakes. These systems are designed to partition and encompass natural planktonic populations to study their responses to chemical (typically pesticide) perturbations (Day et al., 1987; Hanazato and Yasuno, 1989; Kaushik et al., 1985; Solomon et al., 1985, 1986, 1989; Stephenson et al., 1986). Littoral enclosures, enclosures that border the edge of a pond or lake, have been developed and used by the EPA Research Laboratory at Duluth, MN. These systems (5 × 10-m surface area) have been used to study pesticide fate and effects on water-quality parameters, zooplankton, phytoplankton, macroinvertebrates, and fish (Siefert et al., 1989). Brazner et al. (1989) described littoral enclosure construction and endpoints studied and discussed the variability (coefficients of variation) of different indicators.

Pond Systems

Replicated pond mesocosms have been used extensively to evaluate pesticide fate and toxicological effects relationships (Touart and Slimak, 1989). Most ponds used for this purpose are of earthen construction, and their surface areas range in size from 0.04 to 0.1 hectares.

Artificial Streams

In contrast to lentic mesocosms, few attempts have been made to standardize *lotic* experimental systems, even though experimental stream ecosystems have been employed to test chemical effects (see Table 17.1 and Table 17.2). Invariably, the use of constructed stream ecosystems has involved studying the responses to multiple chemicals of a macrobenthic community, including fish, chosen to be "typical" of what might be expected in natural streams. Response variables differ among published lotic studies and obviously depend on the research questions asked and approaches taken (Table 17.2). Relatively few such systems are currently operating in the world. Costs associated with building and operating lotic mesocosm systems often limit the number of experimental units; thus, most stream mesocosm studies

TABLE 17.1

Use of Outdoor Constructed Stream Ecosystems: Physical Parameters

Circulation	Length/ Size (m)	Volume/Flow	Refs.
Flow-through	4.9	77.0 L/min	Dorn et al. (1996, 1997a,b); Harrelson et al. (1997); Kline et al. (1996)
Flow-through	50.0	300.0 L/min	Fairchild et al. (1993)
Partially recirculating	5.0	91.7 L/min	Girling et al. (2000)
Flow-through	110.0	1241.0 L/min	Haley et al. (1995), Hall et al. (1991)
Flow-through	520.0	0.76 m^3/min (0.57 m^3/min, winter)	Hermanutz et al. (1992)

TABLE 17.2

Use of Outdoor Constructed Stream Ecosystems: Chemicals Tested and Response Variables

Chemical	Structural	Functional	Organisms Studied	Refs.
Linear alkyl ethoxylate surfactant	Abundance, biomass	Drift, mortality, growth, reproduction, chlorophyll and pheophytin content	Fish, invertebrates, macrophytes, and periphyton	Dorn et al. (1996, 1997a,b)
Surfactant	Abundance	Mortality, growth, chlorophyll, productivity	Fish and invertebrates	Fairchild et al. (1993)
3,4-Dichloroaniline (3,4-DCA)	Abundance, biomass	Mortality, growth, reproduction, invertebrate drift and behavior, community respiration, and photosynthesis	Fish, invertebrates, and periphyton	Girling et al. (2000)
Effluent	Abundance, biomass, invertebrate diversity	Mortality, growth, histopathology, chlorophyll, and productivity	Fish, invertebrates, and periphyton	Haley et al. (1995)
Effluent	Abundance, biomass	Mortality, growth, histopathology, reproduction, chlorophyll, and productivity	Fish, invertebrates, and periphyton	Hall et al. (1991)
Linear alkyl ethoxylate surfactant	—	Mortality, growth, reproduction, and behavior	Fish	Harrelson et al. (1997)
Selenium	—	Bioaccumulation, mortality, growth, development, reproduction	Fish	Hermanutz et al. (1992)
Surfactant	Abundance	Mortality, growth, reproduction, swimming performance	Fish and zooplankton	Kline et al. (1996)

have evaluated single chemicals at multiple concentrations with or without treatment replication. Designs range from small recirculating streams (Crossland and La Point, 1992) to large, in-ground, flow-through streams 520 m in length (Hermanutz et al., 1992). Most constructed streams are 3 or 4 m in length and about 50 cm wide. Volume flows range considerably and usually are selected to approximate the regional conditions. Artificial stream endpoints selected for study are almost always functional or structural endpoints of algae, benthic invertebrates, or fish (Table 17.2). The size and scale of most artificial streams preclude the use of predator fish, except for very large systems. For short-term studies, pools may be constructed downstream to place herbivorous minnows or larval predators, such as bluegill or bass.

Analysis of Mesocosm Studies

Regression designs are common and suggested for use in risk assessment when experimental units are scarce (Dyer and Belanger, 1999; Shaw and Manning, 1996). Despite problems associated with pseudoreplication (Hurlbert, 1984), lack of replication may be justified because within-unit (e.g., within one replicate unit) variability due to treatments can be substantially more important than among-unit variability (Belanger, 1997). Fewer experimental studies have used factorial designs or addressed issues of multiple stressors (Carder and Hoagland, 1998; La Point and Perry, 1989). Factorial designs that use analysis of variance (ANOVA) (requires replication) are efficient and allow investigation of multiple factor interactions (multiple stressors) (Groten et al., 1996; Underwood, 1997). Table 17.1 and Table 17.2 provide representative examples of experimental designs and endpoints used in outdoor stream mesocosms.

Design Considerations

Several factors must be considered when designing and implementing studies using model ecosystems. Considerations range from the pragmatic (funding, time constraints, etc.) to the heuristic (What are the study goals? What levels of realism are desired?). The physicochemical and biotic features of model systems determine to what extent, if any, the systems represent natural ones. These features also influence contaminant fate and effects. System design is therefore important in defining what inferences may be drawn from the results of tests conducted with surrogate systems and how closely they may be extrapolated to fish populations in natural aquatic ecosystems. Using results from the scientific literature on model ecosystems, the following sections seek to provide a synthesis of some key experimental design considerations.

Scaling Effects in Artificial System Research

The question of whether artificial aquatic systems are reliable surrogates for natural ones is strongly linked to system scale. Scale includes not only the size and physical dimensions of a microcosm or mesocosm but also its spatial heterogeneity and attendant biotic components. Crucial physical and chemical processes behave differently as both a function of, and contributor to, scale; thus, scaling effects can have implications for community structure and the resultant functional attributes of the system. Obviously, long-term studies with fish cannot be conducted in systems that are too small. Careful consideration must be given to not only system complexity but also fish population size (and, thus, its potential to affect the biota). Vital to the research methodology is the choice of spatial and temporal scales in an experiment which may determine whether changes in selected endpoints (i.e., fish community survival) can be detected during a study. Frost et al. (1988) stated that "typically, scale has not been incorporated explicitly into sampling protocols or experimental designs." Appropriate time scales, for example, in model aquatic-system research must be considered when deciding on overall study duration and sampling frequency. The decision regarding how often to sample the fish may have serious ramifications on the ultimate numbers of fish! Temporal sampling should consider life-cycle duration and periodicities of important prey species. When making a decision concerning sampling intervals, one should also consider the temporal behavior of key physicochemical processes, which are often related to pesticide fates and half-lives.

Microcosms, particularly laboratory ones, require little or no equilibration time prior to their use as test systems. Results can be observed quickly, but microcosms are not self-sustaining and tend to become unstable over time. Of course, relative to the species studied, *self-sustaining* and *stable* become relative terms. Because laboratory microcosms can sustain only a limited number of trophic levels, usually composed of small organisms with short lifespans (days to weeks) and rapid turnover times, frequent sampling regimes and short study durations are required. In such systems, it may be appropriate to study larval fish or fish of a few centimeters (perhaps *Gambusia* sp.). Unfortunately, frequent sampling in small systems may damage the system and its biotic contingent (SETAC–Europe, 1991).

System size and overall dimensions may have idiosyncratic implications in the outcome of the project. Dudzik et al. (1979) cited the prevalence of biological and chemical activity on the sides and bottoms of microcosms to be one of the most important problems in microcosm research. Edge effects have been noted and discussed in enclosure studies as well (Arumugam and Geddes, 1986; Stephensen et al., 1984), but the ecological implications of such scaling ramifications in ecotoxicological studies have yet to be resolved. These concerns present a unique challenge in the toxicological arena, as scaling effects may ultimately hinder the validation process, which is becoming increasingly critical in decision and policy making, as well as in enforcement and litigation issues. Edge effects in ecotoxicological work pertain to materials from which littoral and pelagic enclosures are constructed, because the materials may serve as sorption sites for toxins (via adsorption) (Chant and Cornett, 1988; Heinis and Knuth, 1992; Siefert et al., 1990). This problem has been linked to physical scale and system dimensions, as the ratio of wall surface area to water volume is greater in smaller test systems. Smaller enclosures and microcosms may remove disproportionate amounts of pesticide from the water column via absorption to container walls (SETAC-Europe, 1991). A study investigating the role of spatial scale on methoxychlor fate and effects in three sizes of limnocorrals found pesticide dissipation was more rapid than expected in the smallest enclosures (Solomon et al., 1989). These findings were associated with less severe impacts and quicker recovery of zooplankton populations in the smallest enclosures; hence, the success of such studies is, in part, contingent on understanding the role of spatial factors in biotic organization. To address such concerns, Stephenson et al. (1986) studied the spatial distributions of plankton in limnocorrals of three sizes (equal depths), in the absence of perturbations, to assess the viability of such systems in community-level toxicant research. The most predominant edge effects were reported in the largest enclosures, where macrozooplankton occurred in significantly higher numbers than did microzooplankton.

The limited size and accompanying physical homogeneity of smaller mesocosms create additional problems for their use with fish populations: (1) They are particularly susceptible to stochastic, often catastrophic, events from which system recovery may be highly variable relative to larger mesocosms (SETAC-Europe, 1991), and (2) the often limited species compositions of microcosms induce overly strong biotic couplings, resulting in drastic population oscillations and competitive exclusion events (Landis et al., 1994).

As discussed previously, large outdoor systems such as pond mesocosms, require colonization and equilibration times of months to years because they may incorporate many trophic levels, and an extensive number of interactions occur as a function of greater physical scale. Frequent (i.e., daily) sampling for many selected parameters may not be logistically feasible, or even necessary, to detect effects at the population or community levels. It is an implicit assumption in these studies that fish (top predators in the systems) integrate effects over the study duration. If their prey base is damaged, we may be able to see the consequences in the top predators. Study duration must be sufficiently long, as impacts at higher levels of organization, particularly indirect effects, may not be evident early in the study. Such systems are presumably self-sustaining enough to permit the study periods necessary for detecting effects at these higher levels.

Mesocosm scale must be considered when designing studies using fish because scale will affect the test outcome, whether the experimenter acknowledges it or not. Most researchers are aware of the implications of system size in fate and effects research, although indirect results in these studies may not always be perceived or attributed to their actual causes. Temporal aspects are also recognized, although the interaction of timing and spatial factors remains not well understood. The treatment of these scaling considerations in a more integrated fashion will ultimately enhance the predictive value and ecological relevance of the results.

Variability

Variability is inherent in any biological system. Understanding the sources of variability is critical when the ultimate goal is prediction. Replication of treatments and the use of controls are absolutely critical to distinguish natural variation from treatment effects (Crossland and La Point, 1992). Sampling replication can assess within-system heterogeneity resulting from spatiotemporal variation in community structure and physicochemical parameters. Studies of benthic species responses have indicated that the sample number required to obtain adequate representation of community species composition may be quite high and sometimes impractical (Chutter and Noble, 1966; Dickson and Cairns, 1972; Needham and Usinger, 1956). There is also the risk that accepted sampling regimes in lentic and marine research may similarly underestimate inherent variability in these more homogeneous systems. Using fish as system integrators may lessen variance, as fewer endpoints are assessed. In any case, assessing variability through such methods as coefficients of variation (Green, 1979) and determining the number of sampling replicates to ensure representative sampling become critical in ecological research. Green (1979) emphasized the importance of conducting pilot studies in ecological research and having adequate replication, both in treatment and sampling. Unfortunately, even though the number of replicates required to detect changes of a given magnitude can be determined *a priori*, such estimates do not always match the availability of research resources, personnel, space, or time (Pratt and Bowers, 1992). Sampling, therefore, must focus on those variables that convey scientific meaning and provide investigators with resolving power for detecting differences. Currently, these variables are primarily structural (Schindler, 1987).

In any manipulative experiment, an explicit assumption is that observed effects (i.e., significant differences) are due to the manipulative treatments. Often, however, observed differences among treatment levels, or even among replicates within a treatment level, are influenced by factors other than those being tested (Ellersieck and La Point, 1995; Hurlburt, 1970). When this occurs, it is impossible to separate the covariates, and the hypotheses being tested at the onset may be invalidated. Variability among systems is a frequent contributor to this phenomenon. Sources of variance may be structural, physicochemical, or biotic. Biotic variability occurs at a variety of levels within ecosystems and markedly affects system-level processes such as productivity and respiration. Variance stems from differences among systems prior to study initiation or result from changes that occur during the study. Hurlbert (1970) discussed both initial or inherent variability among systems and the temporal changes that occur within systems.

The confounding influence of system variability in ecotoxicological studies involving microcosms and mesocosms has long been recognized (Odum, 1984), but no uniform approach to a solution has been reached. Some researchers (Christman et al., 1994; O'Neil et al., 1990) have attempted to assess inherent variability and determine the amount of sampling replication required to detect treatment effects. Other solutions, particularly if fish reproductive endpoints are the primary interest, involve establishing more stable communities with the expectation that system equilibrium will occur, enhancing similarity among replicates and increasing system realism. Giesy and Odum (1980) suggested that higher trophic levels assert a controlling influence on lower trophic levels in microcosms being used for effects studies. Giddings and Eddlemon (1977) studied microcosm variability for the purpose of determining the validity of using such model systems in toxicological research. Methods of limiting among-system variability sometimes employ design features. One routinely applied method in mesocosm, and sometimes in microcosm, research circulates water among the systems prior to study commencement (Crossland and Bennett, 1984a,b; Crossland et al., 1986; Wolff and Crossland, 1985). Heimbach et al. (1992) developed outdoor microcosms in which three interconnected tanks were joined via wide locks (passageways). Water exchange was allowed during an acclimation period followed by isolation prior to pesticide application. Systematic seeding of the systems with biota and sediments from mature ponds may minimize variability resulting from non-uniform distributions of macroinvertebrates and macrophytes (Howick et al., 1992). Such attempts at achieving a degree of sameness among replicate systems are critical, if one is to ascribe changes in fish growth or reproduction to the dosing regime.

Colonization and Acclimation

Ecological maturity of mesocosms affects the degree of variability of both physicochemical and biological parameters used to investigate the impact of contaminants (Caquet et al., 2000). The establishment

of biological organism communities is a critical part of microcosm and mesocosm experiments. Adequate time is required to establish a number of interacting functional groups (Giesy and Odum, 1980). Colonization methods used in microcosm and mesocosm research vary as a function of system size, type of study, whether the study is fate or effect oriented, and the endpoints of interest (Kennedy et al., 1995). Studies using limnocorrals and littoral enclosures usually have no acclimation period because it is assumed that these systems enclose established communities (with or without fish) (Lozano et al., 1992; Solomon et al., 1989). In stream mesocosms, stabilization periods of 10 days (Genter et al., 1987), 4 weeks (Fairchild et al., 1992), and 1 year (Lynch et al., 1985) have been reported. Duration of the maturation period for pond mesocosms varies from 1 to 2 months to 2 years (deNoyelles et al., 1989). Following initial system preparation, acclimation is usually required to allow the various biotic components to adjust to the new environment and to establish interspecific and abiotic interactions. Duration of acclimation time depends on system size and complexity. Systems with more trophic levels will form more complex interactions that require more time to equilibrate than do small systems with fewer species. The time required to equilibrate will increase with initial system complexity, although the use of natural sediments usually shortens the duration of the stabilization period because natural maturation processes are enhanced (Kennedy et al., 1995). During this acclimation period in outdoor systems, the initial preparation of the systems is typically controlled, and natural colonization by insects and amphibians contributes to biotic heterogeneity and system realism. Continuous colonization, however, presents further problems in that each system tends to follow its own trajectory through time. These trends are most apparent in small-scale systems and in systems that have been in operation longer (Heimbach et al., 1994). Circulation of water between the different systems has frequently been proposed as a way to limit among-system variability during this period (Crossland, 1984; Crossland et al., 1986).

Macrophytes

Aquatic vascular plants play a key role in system dynamics within natural lakes, and their presence in model ecosystems makes them more representative of littoral zones in natural systems; however, once introduced into model ecosystems, macrophyte growth is difficult to control and may vary greatly among replicates. This is of particular concern in field studies because macrophytes influence chemical fate, occurrence, and spatial distribution of invertebrates and growth of fish. Bluegill sunfish, for example, require vegetated areas to nest for spawning purposes, and a proper test system, if field responses on bluegill reproduction are important, must take nest habitat into consideration. Thus, variations of plant density and diversity in model ecosystems contribute to system variability and must be accounted for. Macrophyte density affects chemical fate processes by increasing the surface area available for sorption of hydrophobic compounds. The pyrethroid insecticide deltamethrin accumulated rapidly in aquatic plants and filamentous algae during a freshwater pond chemical fate study (Muir et al., 1985). Caquet et al. (2000) measured residues of deltamethrin and lindane in macrophyte samples for 5 weeks after treatment but never in the sediment. Macrophytes also affect physicochemical composition in surrounding waters, influencing the distribution of many aquatic prey organisms (Barko et al., 1988). In addition, macrophytes provide a three-dimensional structure within constructed ecosystems that affects organism distribution and interactions. Brock et al. (1992), in a study with the insecticide Dursban 4E, observed considerable invertebrate taxa differences between *Elodea*-dominated and macrophyte-free systems. Other workers have shown macroinvertebrate community diversity to be influenced by patchy macrophyte abundance (Street and Titmus, 1979) and specific macrophyte types (Learner et al., 1989; Schramm et al., 1987). Cladoceran communities are also associated with periphytic algae on aquatic macrophytes (Campbell and Clark, 1987).

Impacts of chemicals on macrophyte densities indirectly affect organisms by influencing trophic linkages such as predator–prey interactions between invertebrates and vertebrates. Bluegill utilization of epiphytic prey may be much greater than predation upon benthic organisms (Schramm and Jirka, 1989). Excessive macrophyte growth may force fish that normally forage in open water to feed on epiphytic macroinvertebrates where energy returns are not as great (Mittlebach, 1981). Fish foraging success on epiphytic macroinvertebrates depends on macrophyte density (Crowder and Cooper, 1982) and plant growth form (i.e., cylindrical stems vs. leafy stems) (Dionne and Folt, 1991; Gilinsky, 1984; Loucks, 1985). Dewey (1986) studied atrazine impacts on aquatic insect community structure and emergence

and fish community structure. Decreases in the number of insects in this study were correlated with reductions in aquatic macrophytes and associated algae and increases in fish predators. Insect responses were not a direct effect of the pesticide. The wide range of chemical, structural, and biotic interactions dependent upon macrophyte type and density, as outlined above, emphasizes the central role of this part of the community in lentic systems. It is apparent that the design of surrogate ecosystems needs to consider plant density and diversity as a contributor to system variability and the inability to detect ecosystem changes.

Fish

Whether to include fish, what species or complex of species to select, the loading rates, and their potential for reproduction are critical factors to consider in experimental design. Fish populations are known to have direct and indirect effects on ecosystem functioning. Fish predation is known to alter plankton community composition (Brooks and Dodson, 1965; Drenner et al., 1986; Vinyard et al., 1988), and the presence of fish in limnocorral or microcosm experiments may alter nutrient dynamics and cycling (Mazumder et al., 1988, 1989). During an outdoor microcosm experiment, Vinyard et al. (1988) found that filter-feeding cichlids altered the quality of nitrogen (shifting the dominant form) and decreased limnetic phosphorus levels via sedimentation of fecal pellets. Additionally, unequal fish mortality among replicate microcosms may influence nutrient levels independently of any other treatment manipulations (Threlkeld, 1988). In separate limnocorral studies, Brabrand et al. (1987) and Langeland et al. (1987) concluded that fish predation alters planktonic communities in eutrophic lakes and that the very presence of certain fish species may contribute to the eutrophication process. These studies offered a number of interesting hypotheses regarding fish effects in limnetic systems; unfortunately, the experimental designs of these studies lacked treatment replication, limiting their inferential capability.

Many studies completed in 1986 through 1992 in the United States, under EPA guidelines (Touart and Slimak, 1989) for pesticide studies, required that mesocosms include a reproducing population of bluegill sunfish (*Lepomis macrochirus* Rafineque). Presumably, these fish and their offspring are integrators of system-level processes. Variances in numbers, biomass, and size distribution among different pesticide exposure levels provide requisite endpoints for risk management decisions. Chemical registration studies by Hill et al. (1994b), Giddings et al. (1994), Johnson et al. (1994), Morris et al. (1994), and Mayasich et al. (1994) have determined that the abundance of young bluegill in mesocosm experiments obscured or complicated the evaluation of pesticide impacts on many invertebrate populations. This is consistent with Giesy and Odum's (1980) suggestion that higher trophic levels assert a controlling influence on lower trophic levels in microcosms being used for effects studies. Ecological research with freshwater plankton and pelagic fish communities indicates that both top-down and bottom-up influences affect planktonic community structure and biomass (Carpenter et al., 1985; McQueen and Post, 1988; Threlkeld, 1987). These relationships have not been investigated to the same degree in littoral zone communities, and the role of benthic macroinvertebrates in these trophic relationships requires further study. Along these lines, Deutsch et al. (1992) stocked largemouth bass in pond mesocosms to control unchecked bluegill population growth, thereby potentially limiting among-system variability and providing a more natural surrogate system. The desirability of adding bass to mesocosms, however, must be balanced against possible increases in experimental error variances that may result from differential predation on bluegill if variable bass mortality occurs in the ponds (Stunkard and Springer, 1992). The only way to control variability in predation of bluegill would be to maintain equal levels of predator mortality in all ponds. Scaling is important, and criteria for fish stocking levels are highly dependent on system size. Fish population density should not exceed the carrying capacity of the test system. Biomass densities should generally not exceed 2 g/m^3 (Fairchild et al., 1992). It may be useful to stock mesocosms with low adult densities and remove adults and larvae after spawning; however, the life stage, number and biomass of fish added will depend on the purpose of the test. If the emphasis is on an insecticide, for example, larval fish might be added to monitor their growth in relation to the invertebrate food base.

The FIFRA requirement of using a single species (e.g., bluegill sunfish) in mesocosm experiments was very likely not sufficiently protective of natural fish communities for a number of reasons. First, the inherent sensitivity of other fishes compared to bluegill is not known with any degree of certainty. Second,

due to a variety of life-history adaptations, other fish might experience differential exposure to chemicals. For example, surface-dwelling fish, such as topminnows, would potentially be exposed to high initial pesticide concentrations found in the surface layer following treatment. Alternatively, contaminants that sorb to sediments (including many pesticides) might be expected to impact bottom-feeding fish selectively. Drenner et al. (1993) studied the effects of a pyrethroid insecticide on gizzard shad (*Dorosoma cepedianum*) in outdoor microcosms. These fish are filter feeders and commonly have large amounts of bottom sediments and detritus in their digestive systems. This study (Drenner et al., 1993) was unique in its use of nonstandard fish species. Similar field studies utilizing other fish species should be pursued to evaluate the influence of feeding behavior and habitat selection on chemical exposure. Following appropriate research, it is conceivable that a multispecies assemblage (i.e., surface feeder, water-column planktivore, and bottom feeder) might eventually be used to better represent potential impacts to natural fish communities.

Chemical Fate Considerations

It must be acknowledged that the primary use of mesocosm testing for fish is to predict the environmental fate of the chemical. This includes its persistence, its distribution or partitioning among various environmental compartments, and an estimation of its bioavailability and potential to bioaccumulate. (Boyle, 1985). Various chemical characteristics affecting fate are currently measured in the laboratory, such as solubility, octanol–water and soil–water partitioning, and bioaccumulation in different organisms. More comprehensive estimates of the fate of the chemical are manifested in mathematical and physical models of aquatic ecosystems. Boyle (1985) provided a list of different representative types of mathematical models from the literature used to determine the fate of a potential contaminant. Rand et al. (2000) described study design, specific techniques, and the fate of pyridaben (a miticide/insecticide) in microcosms and discussed the usefulness of microcosms to study the fate of a chemical under environmental conditions that are more representative of the field. If the direct effects on growth or reproduction are desired, a laboratory or early-life-stage test can cost effectively provide that information. The use of mesocosms is primarily to show that degradation and the metabolic process limit the exposure and, hence, the availability of the chemical in the environment. Complexity of dosing methods for mesocosm studies varies with purpose of the study. The contaminant may be added to the water surface, to the subsurface, or on the sediments by pouring the active ingredient or adding a mixture of soil and toxicant (Boyle et al., 1996; Cushman and Goyer, 1984; Giddings et al., 1997; Oviatt et al., 1987), spraying with hand-held sprayers and spanners that release the solution onto the water surface (Brazner and Kline, 1990; Crossland, 1982; deNoyelles et al., 1989; Kedwards et al., 1999a; Stout and Cooper, 1983; Sugiura et al., 1984; Ronday et al., 1998), or pumping via a flow-through system (Bakke et al., 1988; Farke et al., 1985; Zischke et al., 1985). Subsurface dosing can also be achieved by placing the spray nozzle or hand-held sprayer below the water level (Boyle et al., 1985). There are nearly as many application methods as there are researchers designing microcosm and mesocosm studies. It should be noted that the method chosen to apply the test material could have considerable influence on its fate and subsequent exposure to organisms; for example, the droplet size from a spray nozzle of an experimental system may differ from the droplet size deposited on a natural body of water following agricultural application to adjacent land. In turn, droplet size may be critical because volatilization from the water surface microlayer can be very rapid and may be a major route of dissipation (Maguire et al., 1989). Thus, the decision of whether a chemical is sprayed on the water surface or injected underneath can have a major influence on its half-life. Clearly, the method of test material application must be chosen so realistic exposures are obtained.

Experimental Design and Statistical Considerations

Experimental Design Considerations

Key issues in designing microcosm and mesocosms tests are treatment replication, sample size and power, optimization criteria in design selection, choice of number and spacing of dose levels, inference on safe dose, and defining the dose–response curve (Smith and Mercante, 1989). Biological variables

measured in field studies have a large variance associated within and among test systems that can decrease the ability to detect ecosystem effects (Kennedy et al., 1999). One approach to improving designs is by reducing variation. Although this may be accomplished by increasing the number of microcosms, costs will ultimately limit the number of replicates possible. Information gathered through power analysis can optimize resources and expenditures to produce the best possible experimental or sampling design and determine which biological parameters should be included in a study protocol (Kennedy et al., 1999).

Endpoint Selection

Toxicological endpoints derive from specific measurements made during or at the conclusion of toxicity tests (Adams, 1995). For purposes of ecological risk assessment, two types of endpoints have come into common use: assessment and measurement endpoints. Assessment endpoints refer to the population, community, or ecosystem parameters that are to be protected, such as population growth rate or degree of eutrophication (Suter, 1995). Measurement endpoints refer to the variables measured, often at the individual level, that are used to evaluate the assessment endpoints. Measurement endpoints describe the variables of interest for a given assessment. Common measurement endpoints include descriptions of the effects of toxic agents on survival, growth, and reproduction of single species. Other measurement endpoints include descriptions of community effects (respiration, photosynthesis, or diversity) or cellular effects. In each case, the measurement endpoint is quantitatively measured and used to evaluate the effects of the toxic agent on a given individual, population, or community. Sometimes it is not possible to examine an assessment endpoint directly. In such a case, measurement endpoints are used to describe the organism or entity of concern (Suter, 1995). The underlying assumption in making toxicological endpoint measurements is that the endpoints can be used to evaluate or predict the effects of toxic agents in natural environments. Suter (1995) discussed endpoints appropriate for different levels of organization, and EPA risk assessment guidelines (USEPA, 1992) provide information on how endpoints can be used in the environmental risk assessment process. An innovative stream mesocosms study (Dube et al., 2006) used egg production and number of spawning cycles in pearl dace (*Semotilus margarita*) as endpoints. The authors found that metal mine effluents significantly decreased egg production and reduced the number of spawning cycles. Exposure to the mine effluents in these flowing systems also caused significant mortality in the F_1 generation. Despite the interest in and usual use of meso- and microcosms for single-species endpoint purposes, fish will influence the results of their prey (Sanchez-Bayo and Goka, 2006; Van den Brink et al., 2005). Subtle responses to chemicals (particularly those at very low environmental concentrations) may be masked by the biological interactions among predators and prey; however, food chain indirect effects can be tested by a proper selection of the assembled species (Van den Brink et al., 2005).

Species Richness, Evenness, Abundance, and Indicator Organisms

The presence of species and their relative abundances is used as a measure of the degree of contamination of an aquatic habitat (Lamberti and Resh, 1985; Sheehan, 1984). These parameters are often used to calculate diversity indices. Although diversity indices have been shown to be insensitive to slight to moderate perturbations (Barton, 1992; Cao et al., 1996), they are still reported in biological monitoring. (Camargo, 1993; Joshi et al., 1995). Species richness (the number of different species and evenness of the distribution of individuals among the species present) has been shown to better reflect impacts to aquatic communities than diversity indices (Dickson et al., 1992). The abundance of species has been a standard measure for good-quality habitat since early studies of habitat perturbation (La Point, 1995). For studies in which the chemical may sorb to sediments, the meiofauna (such as nematodes and ostracods) become important links in benthic food webs (Hoess et al., 2004). Meiofauna are an important functional group feeding on bacteria; however, changes in numbers or biomass of nematode communities may be responding to biotic or abiotic components in the mesocosms, and further studies are required to confirm the benefit of quantifying nematode abundance (Hoess et al., 2004).

Univariate Methods

Univariate techniques, particularly ANOVA, using parametric or log(x + 1) transformed data, are commonly used in testing fish population endpoints, with either Dunnett's or the Student–Newman–Keuls (SNK) method serving as common *post hoc* tests (Graney et al., 1994). Linear regression and correlation have also been used, but with less frequency (Liber et al., 1992). When assumptions of parametric tests, normality, and homogeneity are not met, nonparametric tests such as Spearman's coefficient of rank, the Wilcoxon rank sum statistic, or the Kruskal–Wallis test should be used. Whereas univariate methods of hypothesis testing may be appropriate for single-species endpoints (e.g., bass reproduction over the season), univariate statistics are inappropriate for multispecies toxicity tests. Often, in regulatory tests, univariate endpoints are an attempt to understand multivariate systems by looking at univariate projections, attempting to find statistically significant differences in a key endpoint of interest. In a regression ANOVA approach, an effective concentration can be determined and used, with caution. The tests are quite site and situation conditional. Calculated no-observable-effect levels (NOELs) or lowest-observable-effect levels (LOELs) depend on the statistical power and concentrations chosen (Graney et al., 1994). Such limits are functions of the experimental design rather than components of the intrinsic hazard of the chemical being studied. When data are analyzed by ANOVA, Knauer et al. (2005) suggested that it might be possible to use different significance levels for abundant and for less abundant species.

Multivariate Methods

Large variances are common in aquatic mesocosm studies. In such situations, the statistical power required to detect effects can be sufficiently low such that the usefulness of the analysis is questionable. Even if effects exist, they may not be detected (Peterman, 1989); however, even if univariate procedures are performed with satisfactory power, interactions among species, populations, or communities are usually not considered (Kennedy et al., 1999). Multivariate techniques offer potential solutions to these analytical and interpretational problems (Sparks et al., 1999). Analyzing ecotoxicological field studies with multivariate techniques has some clear advantages. Whereas fish population dynamics may be an appropriate endpoint, community-level approaches have more ecological relevance than do studies of individual populations isolated from their environment. Multivariate statistics analyze all available data and are more likely to discriminate among treatments; consequently, such approaches may help to determine the ecological significance of chemical exposure and may reach conclusions based on ecological significance, a fundamental responsibility in field studies (Crossland and La Point, 1992; Van den Brink and Ter Braak, 1999). Multivariate techniques are also ideal for handling large amounts of data and endpoints more effectively. Kedwards et al. (1999b) showed how multivariate techniques aid in the interpretation of biological monitoring studies which otherwise present difficulties related to the sometimes semiquantitative nature of the data and the unavailability of true control sites, replication, and experimental manipulation.

Several books detail the methods of multivariate analysis: Ludwig and Reynolds (1988) provided an introduction to the assumptions, derivations, and use of several multivariate techniques commonly used for the analysis of ecological communities. Van Wijngaarden et al. (1995) compared detrended correspondence analysis (DCA), principal components analysis (PCA), and redundancy analysis (RDA) and their usage in mesocosm research in more detail. Van den Brink et al. (1996) proposed a multivariate method based on RDA, and Clarke (1999) demonstrated the use of nonmetric multivariate analysis in community-level ecotoxicology, which does not require the restrictive assumptions of parametric techniques. Multivariate techniques have become more accessible and user friendly with the availability of software such as the principle response curves method (Van den Brink and Ter Braak, 1999) and routines in other software packages. Major steps have also been taken to produce outputs readily interpretable by both ecologists and environmental managers and regulators. Multivariate techniques now provide the ecotoxicologists with powerful tools to visualize and present impacts at the community and ecosystem level.

Summary

Fish species tested in experimental ecosystems are assumed to reflect changes in their environment. In some systems, this may be true; however, the success in using such systems depends on establishing appropriate temporal and spatial sampling scales. As artificial ecosystems must be sampled with response times for species taken into account, sampling programs should reflect the variance in activities, life spans, and reproductive potential of the fish species of interest. This can call for long-term experimentation, particularly if the fish species of interest are larger, typically predatory organisms (e.g., bluegill sunfish, trout, bass). When performing a model ecosystem study, it is important to determine the ecological relevance of effects identified in linked laboratory studies. Interpretations of field studies often focus on effects at the community level, with the assumption that the top predators integrate lower-species responses. This assumption needs to be further tested. A second reason for conducting studies in model ecosystems is to measure the fate and distribution of the chemical under environmentally realistic exposure conditions. Chemical partitioning to sediments and plants as well as photolysis and other processes influence chemical fate; moreover, mesocosm studies incorporate natural abiotic conditions (e.g., temperature, light, pH) that influence organismal responses. An aspect of testing chemicals under semirealistic conditions is that predicted environmental concentrations of nonpersistent chemicals will usually overestimate the real and effective concentrations to which organisms are exposed in their natural environments (Lam et al., 2004; Richards et al., 2004; Sanchez-Bayo and Goka, 2006). The design of mesocosms studies must take into account chemical dissipation to better predict impacts. Lam et al. (2004) noted that "given the complexity of interactions within the food web, it is almost impossible to predict the side effects (e.g., the domino effect) that may result from toxicological disturbances, such as the application of pesticides to agroecosystems or pollutants in the wider environment." We agree with that cautionary note.

There is no single best experimental design or test system, as a number of options are available, depending on budgets and facilities. The experimental design must address the study objectives and characterize the distribution and fate of the chemical under study and the ecosystem potentially affected. Historically, mesocosm tests have been viewed as a continuation of single-species tests (the regression–ANOVA approach is currently favored). Typically, one species (the top predator) is studied. Methods employing multivariate statistics to evaluate endpoints in a more integrated and holistic fashion should be applied to these studies. Multivariate techniques should not be viewed as a panacea for data analyses (James and McCulloch, 1990) but should be part of an integrated approach that encompasses both empirical and modeling approaches. Ultimately, the value of research using surrogate systems lies in the potential of these systems to provide probabilistic predictions of ecosystem responses to contaminants.

Mesocosm studies using fish as endpoints, to be effective contributors to the ecological risk assessment process, present an exciting and challenging problem. To be successful, this task will require novel approaches that integrate basic ecological principles, toxicology, and statistics; however, study design for ecotoxicological research goes beyond just innovative science. A mesocosm study is not an end in itself. Ultimately, results of these studies will be used as one assessment tool to ensure that the risk of pesticides to human societies is minimal. As a result, it is critical that mesocosm study protocols consider the endpoint needs of the regulatory agencies and risk assessment managers. Finally, the mesocosm researcher must have a clear *a priori* understanding of how enclosure results will scale up to perform landscape-scale risk assessments. If these tasks are considered during the study design phase, the probability of a successful mesocosm study is strongly enhanced.

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18

Ecological Risk Assessment

David R. Mount and Tala R. Henry

CONTENTS

Introduction

Ecological risk assessment has been defined as "a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors" (USEPA, 1992). Conceptually, risk assessment is implied by Paracelsus' observation that "only dose differentiates a remedy from a poison." Risk is not an inherent property of a chemical toxicant but rather the product of its toxicity and the exposure received. Hence, the same chemical can show little risk at low exposures, but high risk at high exposures. This basic concept is embedded in our everyday behaviors. Gasoline is potentially very toxic to human beings, but we handle it without a thought on an almost daily basis. Why? Because even though gasoline has high toxicity, we have decided, consciously or unconsciously,

that there is little chance that we would be exposed to that gasoline in a way and quantity that would cause us serious harm. It is this weighing of exposure and potential effect that is, in essence, a form of risk assessment.

Although we each evaluate risks many times each day, the actual process we use to judge risk is probably something we would struggle to explain. Moreover, different people assess risks differently and choose different behaviors as a result, but we would also struggle to articulate exactly how their risk assessment process differs from our own. When it comes to environmental decision making, we intuitively expect something more transparent and objective from regulatory authorities and other decision makers. The need for an objective and consistent framework for judging environmental risks has led to the process we know today as ecological risk assessment.

Through the years, the term *ecological risk assessment* has been coined to refer to risk assessment applied to nonhuman ecological receptors (e.g., fish, terrestrial wildlife, plants) or more generally to the natural environment; the term *human health risk assessment* is used in reference to assessments focusing on effects to humans. The remainder of this chapter focuses on ecological risk assessment in its broadest definition, reviewing the evolution of the concepts into a formalized scientific process and describing how the science of toxicology is woven into the risk assessment process. The primary context is ecological risk assessment for chemical pollutants, although the principles are also generally applicable to other stressors.

The Evolution of Environmental Toxicology and Ecological Risk Assessment

Regulatory decision making has been a major impetus in the development of the process of ecological risk assessment, presumably because it is in this context that our basic understanding of the effects of chemicals on organisms must be organized into a framework for making quantitative decisions about the ecological acceptability of chemical exposures in the environment. Looking retrospectively at major developments in environmental toxicology and regulation within the United States, one can see the emergence of many concepts important to ecological risk assessment today.

In the 1960s, Mount and Stephan (1967) proposed the *maximum acceptable toxicant concentration* (MATC), which is one of the earliest examples of a standardized process for describing toxicity in the context of risk. The MATC was developed to describe the results of aquatic life-cycle toxicity tests and was defined initially as the range between the highest toxicant concentration that did not cause an adverse effect on any tested endpoint (i.e., survival, growth, or reproduction) and the lowest test concentration that did cause an adverse effect. Later, this range was abbreviated to a single concentration, the geometric mean of these values (often called the *chronic value* today). Although the concept itself is still in use, the very term MATC implies more than an expression of risk (how much effect is induced by an exposure), but also an expression of the acceptability of that risk (how much toxicant is allowable). As is described later in this chapter, current risk assessment practices segregate these elements. Nonetheless, the MATC concept marked a significant move forward in establishing a consistent means to relate chemical exposures to expected effects. It is interesting to note that the same chronic effect endpoints used to derive the original MATC are still in widespread use but have been renamed the *no-observed-effect concentration* (NOEC) or *no-observed-effect level* (NOEL) and the *lowest-observed-effect concentration* (LOEC) or *lowest observed effect level* (LOEL). This newer terminology does, in fact, focus on an expression of risk (projected biological effects) rather than environmental acceptability.

In 1977, a workshop was held in Pellston, Michigan, for the express purpose of gathering experts in aquatic toxicology and environmental fate for a discussion of how to structure an assessment framework for evaluating risk (then called *hazard*) to aquatic life posed by chemical substances (Cairns et al., 1978). The proposed structure is very similar to the structures used for risk assessment today and provides a succinct conceptual representation of the process. As illustrated in Figure 18.1, chemical exposure is represented by an *expected environmental concentration* (EEC) and is compared to a threshold for unacceptable biological effects, which can be thought of as a *safe environmental concentration* (SEC). Each of these values has a degree of uncertainty about it, which is inversely proportional to the amount of available data. When few data are available, uncertainties around one or both of these values may be

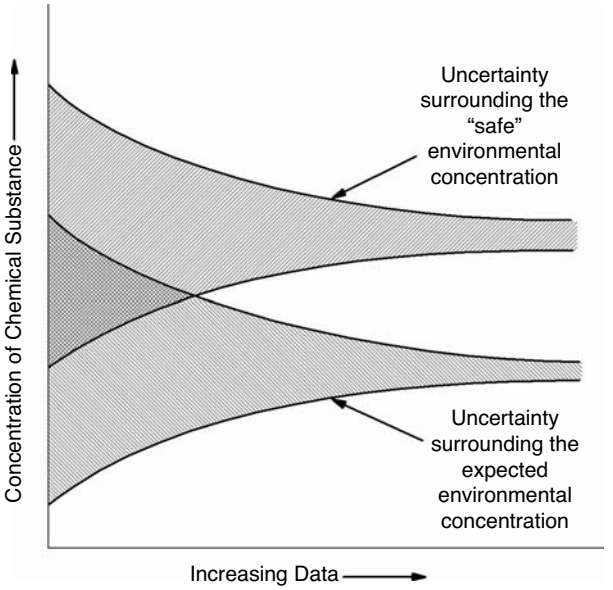

FIGURE 18.1 Conceptual representation of risk (hazard) assessment process. (Adapted from Cairns, Jr., J. et al., Eds., *Estimating the Hazard of Chemical Substances to Aquatic Life*, ASTM STP 657, American Society for Testing and Materials, Philadelphia, PA, 1978.)

large, so large that one cannot discern whether the intensity of exposure is actually greater or smaller than the threshold for unacceptable effects. With the collection of additional data, uncertainties around the EEC and SEC can be reduced until their relationship can be determined. A notable feature of this concept is that the degree of certainty sufficient to allow a decision is not fixed but is dependent upon the distance between the EEC and SEC. Cases where expected exposure and effect concentrations are very close may require extensive study, while situations where these concentrations are far apart may allow decisions to be made with a minimum of data. Figure 18.1 is only a very generalized view of a complicated process, but the concept of iterative data collection and analysis is a cornerstone of the tiered data collection/evaluation schemes that are widely used for environmental decision making.

Through the late 1970s and early 1980s, the establishment of procedures for developing *ambient water quality criteria* (AWQC) by the U.S. Environmental Protection Agency (USEPA) included further refinements in processes used to quantify (potential) ecological risk. AWQC are intended to define chemical concentrations below which the intended uses of water bodies (e.g., drinking water, supporting aquatic life, agricultural use) can be sustained. Early iterations of AWQC were derived using expert panels to develop guidelines using whatever data were available and best professional judgment (USDOI, 1968; USEPA, 1973, 1976). Beginning in 1978, however, the USEPA began developing specific procedural guidelines for the derivation of AWQC to protect aquatic life (USEPA, 1978, 1980, 1985). These guidelines specified a minimum dataset necessary to derive AWQC, requiring that toxicological data be available for at least eight species of organisms with a specified phylogenetic distribution. A statistical procedure was used to estimate the 95th percentile of the species sensitivity distribution, and this, along with chronic toxicity information, was used to derive time-weighted average concentrations which, if not exceeded more than once every 3 years, would not be expected to cause unacceptable adverse effects on aquatic organisms. This approach provided some significant advances with respect to ecological risk assessment, particularly in the areas of uncertainty and probability. Requiring a minimum species coverage in the toxicity data helped to quantify variability (and therefore uncertainty) in species sensitivity and created some consistency in estimates across different chemicals. Probability began to be considered in the context of the statistical distribution of species sensitivity. Protection of entire aquatic communities was clearly intended, grounded in the presumption that by preventing effects on individual species, degradation of the community as a whole would also be prevented.

Further advances in assessment approaches were contained in the development of water-quality-based effluent permitting in the early 1980s. Effluent discharges in the United States are permitted under the National Pollutant Discharge Elimination System (NPDES). Through the 1970s, discharge requirements were largely technology-based standards derived from the level of effluent quality that could be reasonably achieved by each industrial or municipal discharger using the treatment technologies available at the time. Although substantial reductions in pollution discharges were achieved, these standards did not directly assess or prevent potential risk to organisms in the receiving waters. As such, there was no assurance that meeting technology-based performance standards would actually protect aquatic life.

To address this shortcoming, the USEPA developed a water-quality-based approach to effluent permitting that focused on establishing permit limits that would prevent exposures in the actual receiving water from exceeding thresholds for effects on aquatic life (USEPA, 1984, 1989, 1991). In its simplest form, this approach used mass balance calculations to determine the maximum amount of chemical that could be discharged and still maintain chemical concentrations in-stream that met applicable water quality standards to protect aquatic life (frequently the AWQC) or other designated uses. Although theoretically sufficient to protect aquatic life, this mass balance approach does not consider a number of real-world factors such as variability in effluent composition, variability in stream flow, and the assumption that some level of infrequent exceedance of the water quality standard could likely be tolerated by the aquatic community without unacceptable long-term ecological damage. A more advanced analysis was developed (USEPA, 1991) that expresses variation in effluent composition and dilution water flow probabilistically, allowing permit limitations to be established such that water quality standards would be exceeded at less than a specified frequency with a specified level of confidence (e.g., 95% confidence of exceedances less than once every 3 years). This incorporation of variability and probability is very similar to the probabilistic risk assessment approaches that represent the leading edge of the science today.

Ecological Risk Assessment: Definitions and Concepts

While the practical needs of environmental decision making were advancing approaches that would form the foundation for risk assessment, parallel efforts were underway to formalize the concepts underlying risk assessment and to establish a consistent terminology across risk assessment applications. This has led to the four-component framework known today as the *risk assessment paradigm*.

Risk Assessment Paradigm

In 1983, the National Research Council (NRC) defined a framework for risk assessment to facilitate the development of uniform technical guidelines for conducting risk assessments (NRC, 1983). Although this initial framework defined the process exclusively for human health risk assessment, the NRC expanded the framework in 1993 to include ecological risk assessment (NRC, 1993). Different authors and sources vary in detailed definitions, but essentially all current descriptions of both human health and ecological risk assessment contain the four basic components as defined by the NRC (1993): hazard identification, exposure assessment, exposure–response (effects) assessment, and risk characterization. Following the NRC's recommendation that federal agencies develop guidelines to describe how their risk assessments are conducted (NRC, 1993), the USEPA developed *Guidelines for Ecological Risk Assessment* (Figure 18.2) (USEPA, 1998). Although the USEPA's guidelines are not regulations, and risk assessors outside of the USEPA are not obliged to use them, they do follow the NRC framework and provide a comprehensive presentation of the four components of the ecological risk assessment process (Norton et al., 1995; USEPA, 1998).

Hazard Identification and Problem Formulation

Hazard identification (NRC, 1993) and *problem formulation* (USEPA, 1998) are terms used to describe the process of identifying and defining the hazards to be assessed. The NRC framework defines *hazard identification* as "the determination of whether a particular hazardous agent is associated with health or

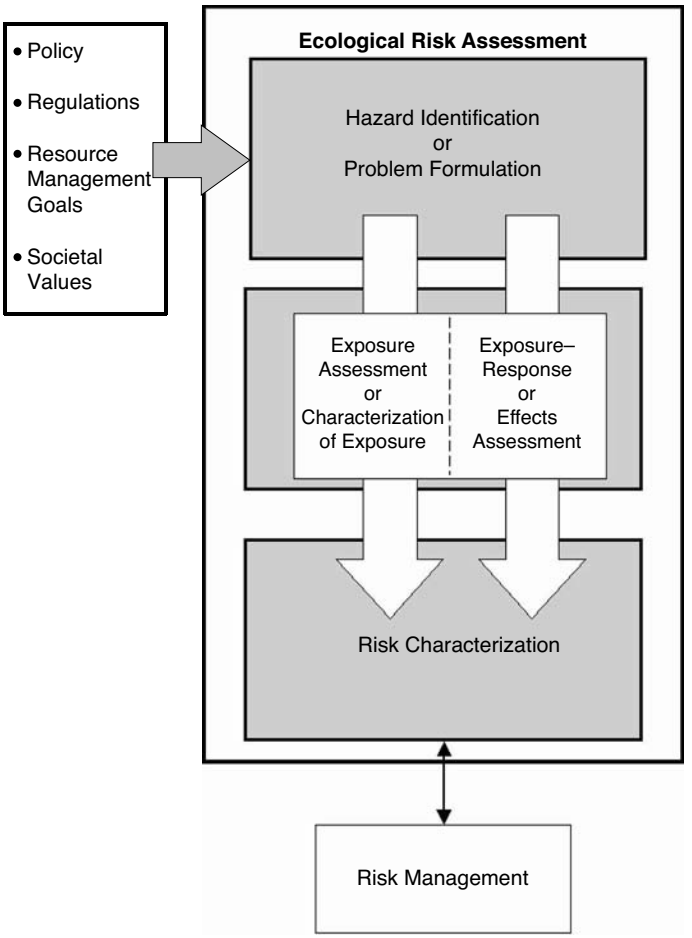

FIGURE 18.2 Elements of the risk assessment paradigm. (Adapted from USEPA, *Guidelines for Ecological Risk Assessment*, EPA/630/R-95/002F, Office of Research and Development, U.S. Environmental Protection Agency, Washington, D.C., 1998.)

ecological effects that are of sufficient importance to warrant further scientific study or immediate action" (NRC, 1993). The USEPA guidelines provide detailed discussion of the types of activities that constitute the problem formulation component of an ecological risk assessment. They suggest that the end product of problem formulation be a conceptual model that identifies and preliminarily characterizes the ecosystem, the stressors known or suspected to be present, the ecological resources to be evaluated (protected), the effects that will be considered, the data needed, and the methods and analyses that will be used. Hence, the problem formulation is a detailed planning document, or road map, for conducting the remaining phases of the risk assessment.

Problem formulation is a critical component of the risk assessment because it defines the scope of the risk assessment and the types of data that will be relevant to assessing risk. In problem formulation, the ecological resources to be evaluated are defined, along with the routes by which chemical exposure and consequent effects may occur and the methods by which they will be measured. The term *assessment endpoint* is used to describe the ecological values (e.g., uses, qualities) for which risk will be assessed. An assessment endpoint may be defined narrowly (e.g., population density of a rainbow trout population in a river reach) or broadly (e.g., healthy and diverse community of native aquatic organisms); however, a broad definition (e.g., a healthy and diverse aquatic community) is subject to many interpretations and may require supplementation with more explicit descriptors of what constitutes "healthy and diverse" in this context (e.g., ecological indices of diversity and abundance).

Many assessment endpoints are either difficult to measure directly or have responses to the stressor that are not known. In these cases, other measures of effect (called *measurement endpoints* in some texts) are used as indicators of the response for the assessment endpoints. For example, in an ecological risk assessment for a zinc-contaminated river, the direct relationship between zinc exposure and trout density may not be known; however, the toxicity of zinc to rainbow trout measured in laboratory tests might be used as a measure of effect for the assessment endpoint of maintaining a self-sustaining trout population (see Figure 18.3). Hence, defining the assessment endpoints and determining the types of data that will be used to evaluate effects on them are essential to ensuring that the appropriate data are collected during the exposure and effects assessment portions of the risk assessment.

In ecological risk assessment, the selection of assessment endpoints is often influenced by both scientific and management (e.g., regulatory or legal mandates) considerations; for example, to identify a species that would be suitable for a measurement or assessment endpoint, scientifically one might consider species susceptibility to a contaminant or the types of effects that occur from exposure to a chemical. Management considerations may include whether protection of an entity is mandated by law (e.g., wetlands, endangered species) or whether the goal is to protect the entity from extinction or optimize its availability for human use (e.g., fishery). Problem definition also involves articulation of the stressors that may affect the assessment endpoint. These stressors may be chemical (e.g., copper, DDT), physical (e.g., dewatering, sedimentation), or biological (e.g., invasive exotic species).

Exposure Assessment or Exposure Characterization

Exposure assessment is defined as the characterization of contact between stressors and the ecological entity of concern. This includes the sources of exposure, the factors that control or influence exposure, and the magnitude of exposure in terms of space, time, and intensity (e.g., chemical concentration). Transport and fate pathways are measured or projected to delineate the extent of the influence of the stressor on the ecosystem and to identify the various media that may act as either secondary sources or as exposure points for the assessment endpoints. The exposure point concentration is the concentration of contaminant in the media to which the ecological entity is ultimately exposed. Exposure point concentrations may be determined directly by measurement or indirectly by modeling. In an aquatic risk assessment, for example, where sediments are the primary source of bioaccumulative contaminants, fish will commonly represent an exposure pathway for piscivorous birds or mammals. To determine the exposure point concentration for the bird or mammal, one could directly measure contaminant concentrations in the fish, or one could measure the contaminant concentrations in the sediment and model bioaccumulation of chemical by the fish. Exposure point concentrations are often combined with measures or estimates of duration and frequency of exposure (i.e., contact rates) to determine the magnitude of the exposure to the biota being assessed. Both direct (e.g., uptake/intake of contaminated sediment, water, soil) and indirect (e.g., ingestion of prey items that have accumulated chemicals from contaminated media) exposures may be applicable for a given assessment endpoint and must be considered in the exposure assessment.

Exposure–Response Assessment or Effects Characterization

The objective of the exposure–response assessment (NRC, 1993) or effects characterization (USEPA, 1998) is to describe the relationship between the amount of exposure and the magnitude of the effects observed or expected to occur. Where effects are known to exist, the plausibility that these effects are occurring as the result of the exposure (i.e., causality) is also evaluated. Data from controlled laboratory experiments and field observations may be used to evaluate ecological effects. A variety of exposure–response approaches, such as point estimates (e.g., LC_{50}, EC_{20}), threshold values (e.g., LOEC), or cumulative distribution functions, may be used to describe the exposure–response relationship. These types of exposure–response relationships are typically quantitative, although qualitative relationships may also be used. If the measurement endpoints are not the same as the assessment endpoints, the linkage of measured effects to the assessment endpoints must be articulated. Extrapolations commonly used to provide this linkage include those between species, between responses (e.g., acute to chronic), between laboratory and field, between levels of biological organization (e.g., individual to population),

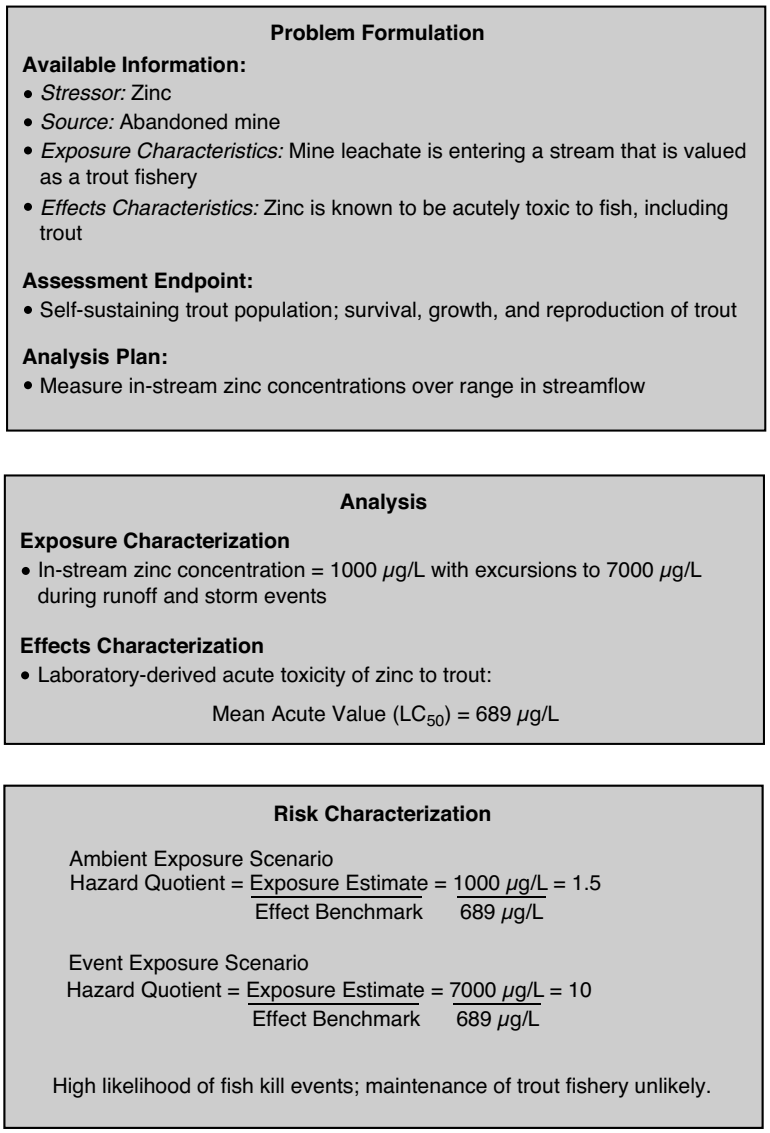

FIGURE 18.3 Example of risk assessment components.

and between geographic locations, spatial scales, and time scales. Extrapolations may be based on empirical models derived from experimental or observational data, on mechanistic models that simulate key processes, or on professional judgment when such information is lacking. The selection of the appropriate exposure–response and extrapolation approaches to be used depends on the scope and nature of the ecological risk assessment and should be defined prior to conducting the effects assessment (i.e., during the problem formulation phase).

Risk Characterization

Risk characterization is the final component of the risk assessment and has as its objective the description of risk in terms of the assessment endpoints identified in the problem formulation. The exposure and effects data are integrated to formulate a risk estimate, typically in the form of a probability or likelihood that adverse effects are occurring or will occur as a result of the existing or projected exposure. Risk

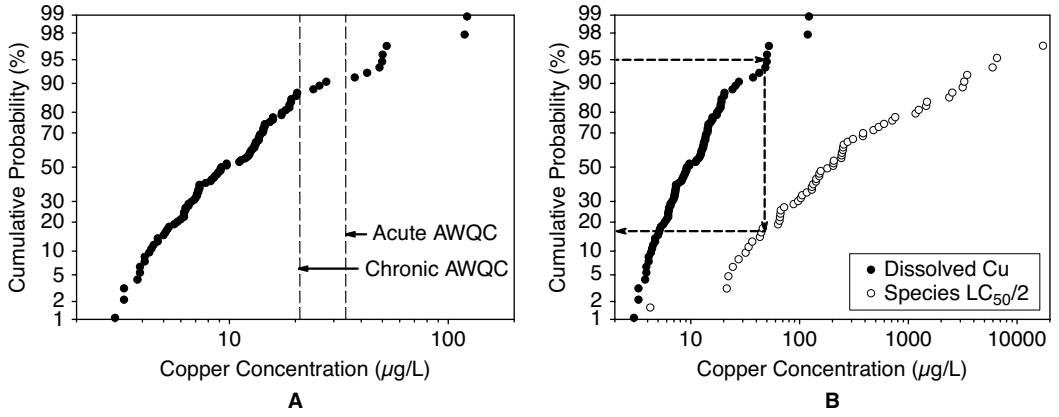

FIGURE 18.4 Rather than choosing single numbers to represent exposure or effect and then calculating hazard quotients, probability distributions can be used to express risk. In panel A, dissolved copper concentrations measured over time in a copper-contaminated river are compared to single-value effect benchmarks (freshwater acute and chronic ambient water quality criteria [AWQC]). This shows the percent of time that concentrations in the river exceed these values (i.e., chronic AWQCs are exceeded about 10% of the time). In a more in-depth analysis, effect benchmarks can also be shown as distributions. Panel B shows the same dissolved copper dataset as it compares to the distribution of acute toxicity thresholds (estimated as the LC_{50} divided by 2) for 62 species of freshwater aquatic organisms. Arrows show that 5% of the time, dissolved copper concentrations exceed the acute toxicity thresholds for about 15% of tested species.

estimates may take a variety of forms depending on the approaches used for the exposure and effects analyses; for example, the use of point estimates for exposure and effects analysis yields deterministic (single-point) risk, whereas risks associated with a range of exposures can be calculated if a full exposure-response curve is available. When single-point exposure and effect estimates are used in risk assessments, relative risk is often expressed as a *hazard quotient* (HQ), which is simply the ratio of the projected exposure concentration divided by the expected effect concentration. HQ values greater than 1 (exposure greater than effect benchmark) suggest that effects might be expected, while values less than 1 suggest the absence of effects. Although appealingly simple, this approach does not lend itself easily to the incorporation of uncertainty (e.g., is HQ = 0.99 clearly a nonproblem and is HQ = 1.01 clearly a problem?). As our ability to quantify expected ecological effects increases, the sophistication of risk assessment procedures is also increasing. Much of the recent effort in ecological risk assessment has focused on developing probabilistic approaches to risk expression, such as "there is a 90% chance that growth of rainbow trout will be reduced by 20% or more" (see Figure 18.4).

Discussion of uncertainties associated with each component of the risk assessment is also an important element of the risk characterization. The description of uncertainties may be qualitative or quantitative and serves to convey the degree of confidence in the risk estimate (for more on uncertainty, see Uncertainty in Risk Assessment section below). The completed risk characterization, including the risk estimate, assumptions and uncertainties associated with the analysis, and the ecological relevance of the findings, is used as the basis for making risk management decisions as well as for communicating risks to interested parties and the public (see Figure 18.5).

Risk Management

Output from the four components of risk assessment discussed above is an articulation of the ecological risks that occur, or are predicted to occur, under the conditions assessed; however, in most situations this expression of risk is not the goal. Instead, the goal is to use this information to help decide how the risks should be managed; for example, are the risks posed by an area of contaminated sediment sufficient to warrant removing the sediments by dredging? The process of evaluating risk information in the context

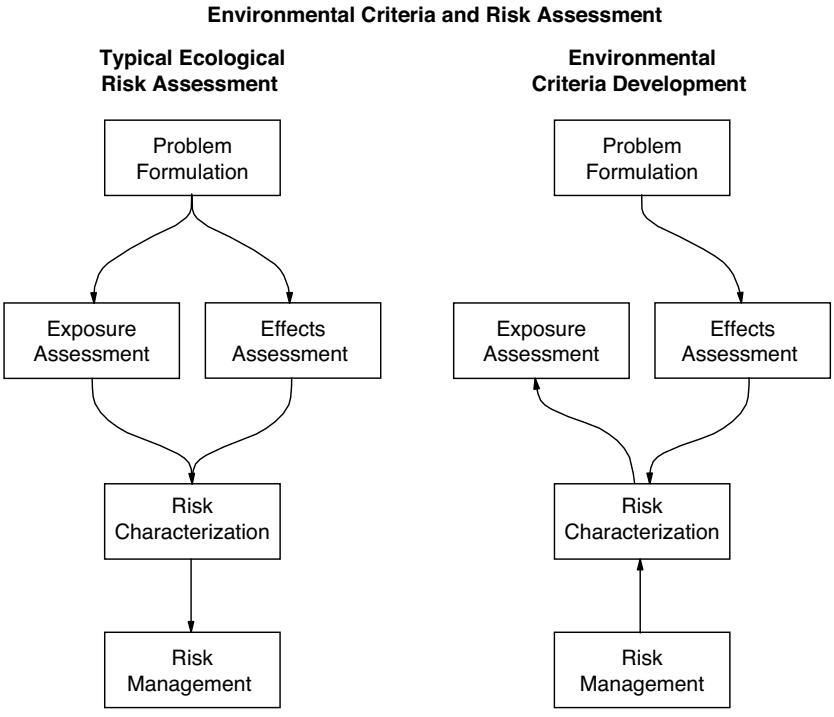

FIGURE 18.5 In the typical application of risk assessment, the analysis flows from problem formulation through exposure and effects analysis, then a prediction of resultant risk is made (left side of figure above). This flow assumes that the exposure of interest is known or can be predicted. In the case of establishing environmental criteria or standards, such as water quality criteria, the exposure is not known *a priori*. Nonetheless, the same principles of risk assessment can be applied, but the flow of the process is changed (right side of figure). As in other risk assessments, one must determine the values to be protected (problem formulation) and the nature of effects caused by the chemical (effects assessment); however, rather than calculating a resulting risk from effects and exposure information, a risk management decision is made to define a threshold between acceptable and unacceptable risk. From this, one can back-calculate the exposure that would equal that threshold condition, which represents the level of exposure that defines the criterion.

of additional factors such as costs and social benefits to reach a decision regarding future actions is referred to as *risk management*. Risk management is the impetus for conducting the risk assessment but is distinct from the risk assessment process (Figure 18.2). The purpose of the risk assessment is to clearly and objectively state the anticipated consequences of an existing condition or anticipated action; it should actively avoid assumptions or conclusions that reflect the personal opinions (biases) of the risk assessor regarding what is environmentally or socially acceptable. Weighing these values is the responsibility of the risk manager. In many risk assessment applications, the risk manager will evaluate several management options, each with its own set of costs (e.g., money, time, disruption) and benefits (e.g., risk reduction, habitat restoration, improved recreational opportunities). In the contaminated sediment example above, these options might be removal by dredging, isolation by capping with clean material, or allowing natural processes to degrade and/or bury the contaminated material.

Comparison of Human Health and Ecological Risk Assessment

Ecological risk assessment has evolved in parallel with human health risk assessment. Although the two are identical in concept and share the same basic structure and process, there are some notable differences in the science necessary to support the assessments.

Hazard Identification or Problem Formulation

In human health risk assessment, the term *hazard identification* is more commonly used to represent this phase of the assessment. Beyond the difference in terminology, the activities undertaken in this component of the risk assessments also vary, primarily in scope. In human health risk assessments, hazard identification is largely related to identification and characterization of the contaminants of concern because the entity (i.e., *Homo sapiens*) and the attribute of the entity (i.e., health) to be protected are largely predetermined. In contrast, in ecological risk assessments ecological risk assessors are often charged with considering entire assemblages or communities of species in selecting and defining assessment endpoints within the context of the contaminants of concern. Human health risk assessments are likely to have less ambiguous management goals (e.g., protection of humans from increased cancer risk at a 10^{-6} probability) than those for many natural resources (e.g., water quality that provides for the protection and propagation of fish, shellfish, and wildlife; Clean Water Act, 1972/1977). This lack of singular ecological management goals reflects the vast diversity in ecological systems and generally increases the complexity of ecological risk assessments and underscores the importance of the problem formulation component. It should be noted that well-defined management goals are not always the result of greater scientific certainty but may simply reflect more well-defined health or environmental policies or societal values.

Assessment Endpoints

Perhaps one of the greatest differences between human health and ecological risk assessment is not intrinsic but rather the result of practical implementation—this difference is in the nature of the risks to be assessed. Although there are no fixed rules about what appropriate assessment endpoints are for human health or ecological risk assessment, widely held societal values have led to a fairly uniform perspective that acceptable risk to humans from involuntary exposure to chemicals in the environment should be essentially zero for all individuals. Although truly zero risk may have relatively little meaning whenever exposure is nonzero, this perspective has led to the need for human health risk assessments capable of addressing levels of risk such as "increased cancer risk less than 10^{-6}," meaning that the acceptable frequency of cancer induced by the stressor is one individual in a million. From the toxicology perspective, this approach has several implications. Not only is protection of single individuals emphasized, but risk levels of one in a million pose a substantial technical difficulty in appropriately extrapolating exposure–response curves generated from experiments conducted using tens to hundreds of observations down to a frequency of one in a million. In contrast, ecological risk assessments generally focus on expression of risk relative to populations or communities of organisms rather than individuals. Thus, the emphasis is not generally on extrapolating to extremely low frequencies of effects on individuals but rather on establishing the rates of mortality, reduced reproduction, or other demographic parameters that will adversely affect the long-term size or structure of the population. The question therefore shifts from "how low must exposure be to have essentially zero effects on individuals" to "how much effect can this population endure before it decreases below an acceptable size?" This difference in perspective greatly influences the science necessary to assess risk.

Extrapolation and Testing

In human health risk assessment, data from several species (e.g., mouse, rat, dog) are often used to extrapolate to a single species (humans), whereas in ecological risk assessments, data for one or a few species must be extrapolated to many species. On the other hand, an advantage in ecological risk assessment is the ability to conduct direct experimentation and collect data directly on the resources that are the subject of the assessment. Data from toxicity tests and field sampling are generally available for many species of fish and wildlife of concern in risk assessments, whereas data for chemical effects on humans are generally limited to epidemiological studies of accidental or inadvertent chemical exposures.

Integration of Human Health and Ecological Risk Assessment

In recent years, efforts have been made to integrate human health and ecological risk assessment processes into a holistic risk analysis approach. In 1997, the USEPA published *Guidance on Cumulative Risk Assessment* to describe an evolution "away from assessment of a single chemical in a single media for causing a particular health effect (e.g., cancer) to assessments in which potentially many stressors in several environmental media may cause or be causing a variety of adverse effects on humans, plants and wildlife or even on ecological systems and their processes or functions" (USEPA, 1997). The document is a first step in providing guidance on how to plan risk assessments that consider multiple stressors (e.g., chemicals, microbial agents, habitat alteration) and exposure media, pathways and routes of exposure, receptors, and assessment endpoints in aggregate to provide a holistic approach to risk reduction (USEPA, 1997). Recognizing the complexity of such risk assessments, the USEPA guidance emphasizes the planning and scoping tasks necessary to accomplish an integrated risk assessment and provides an outline of specific elements that may apply to a particular risk assessment. The elements provided are intended to guide risk managers, risk assessors, and other experts in framing the risk assessment in terms of the sources, stressors, pathways, population, endpoints, and spatial and temporal scale (USEPA, 1997).

The evolution toward integrated risk assessments naturally encourages coordination across statutory mandates and programs or agencies and has thus led to efforts to harmonize both human health and ecological risk assessment processes. The term *harmonization* is used to refer to reconciling or unifying risk assessment processes across toxicity endpoints (e.g., cancer vs. noncancer processes) and among media programs (e.g., water, waste, conservation) and agencies (state and federal). On a broader scale, efforts are also underway to harmonize risk assessment processes globally; for example, the International Programme on Chemical Safety (IPCS), an international cooperative program, has adopted as a specific task the harmonization of approaches for chemical risk assessment (WHO, 2005). The IPCS does not seek to standardize these processes globally but advocates improving the understanding of methods and practices used by various countries in an attempt to promote credible science, efficient use of time and money, and comparison of risk assessments results, as well as to potentially eliminate the need for repeating assessments for the same chemical in various countries.

Applications of Risk Assessment

Risk assessments can be separated loosely into two types, retrospective and prospective, although some assessments contain elements of both. Retrospective assessments seek to quantify effects from past or current exposures. Often these are in essence causality assessments, in that the existence of both exposure and effect are known or suspected, but the degree to which the candidate stressor can account for the observed ecological condition is uncertain. Prospective risk assessments attempt to project risks that will occur from future actions, often weighing the relative risks of alternative scenarios.

As an example of prospective risk assessment, each year approximately 1200 new chemicals are proposed for industrial use in the United States (Auer et al., 1990). Under the Toxic Substances Control Act, the USEPA is responsible for screening these chemicals for potential ecological and human health risks; however, the data requirements under this law are minimal. This presents the challenge of making maximum use of extrapolation tools to make risk predictions using only molecular structure and a small suite of physicochemical parameters. For this reason, structure–activity relationships and other predictive toxicological tools have great utility in this process; however, the need to make risk projections based on a minimum of data exists within the regulated community, as well. Business efficiency provides great incentive for chemical producers and users to identify as early as possible chemicals that are likely to pose unacceptable risks, not only to avoid later liability but also to avoid investing research and development resources in chemicals that will not meet licensing requirements after they are developed. In this context, biochemical and *in vitro* assays have great value as inexpensive indicators of toxicological

properties. Many assessments of this type have large uncertainty because of the limited data but are still effective because they can flag chemicals that either clearly pose substantial potential risk or have a low probability of posing risk if they are developed and used.

Ecological risk assessments conducted in support of contaminated site assessment and remediation generally contain elements of both retrospective and prospective risk assessment; for example, contaminated site assessments under the USEPA Superfund program proceed in two phases: the remedial investigation and the feasibility study. The remedial investigation is a retrospective risk assessment that quantifies the environmental risks posed by current conditions and defines the relationship between the contaminants of concern at the site and the biological effects they cause. The latter is critical to properly evaluating risk management options; if a stream has a severely degraded aquatic community, but stressors beyond site contaminants contribute to that degradation, the relative roles of these stressors will be a factor in deciding what impact site remediation might have. In the feasibility study phase, different approaches for reducing exposure to contaminants and the associated risks are evaluated. For example, for a site with contaminated aquatic sediments, alternatives might include dredging with disposal in an offsite, lined landfill; capping the sediments in place with clean material; or allowing natural chemical breakdown and burial processes to reduce exposure over time. In addition to evaluating the costs, logistics, and impacts of these alternatives, risk assessment is used in a prospective way to forecast the changes in risk that would occur as a result of each action, along with any new risks that might be introduced. The site manager then conducts a cost–benefit analysis to determine which remedial alternative has the combination of costs, impacts, and risk reduction most appropriate for the site (risk management).

Uncertainty in Risk Assessment

All risk assessments involve uncertainties of many types, and evaluating and communicating uncertainty are important but often difficult components of risk assessment; this topic alone is the subject of entire books (Warren-Hicks and Moore, 1998). Different authors have used different schemes for aggregating or parsing the many types of uncertainty using different descriptors. For purposes of this discussion, we will loosely categorize these into: (1) natural stochasticity, (2) parameter error, and (3) model error (Suter, 1993; Suter et al., 1987). Natural stochasticity refers to the natural variation in parameters within the assessment. Examples include variation in chemical exposure caused by natural phenomena (e.g., rainfall events) or differences in sensitivity among individuals in a population. Because these are intrinsic properties of systems, the goal is not so much to eliminate this uncertainty as it is to effectively characterize and incorporate it; for example, rather than representing exposures as single values (e.g., mean exposure concentration), probabilistic models can be used to represent exposures as a distribution of exposures likely to exist over time. Likewise, rather than expressing the potency of a chemical exposure as a single point estimate (e.g., LC_{50}, which is the concentration estimated to cause lethality in 50% of the test population), probabilistic models can be used to predict the percentage of fish that are expected to be affected at any particular exposure concentration. Some of the research needs in this area include developing toxicity testing and analysis methods that make greater use of the entire exposure–response curve (see Figure 18.6). Further, because exposures in most toxicity tests are constant and those in nature are generally variable (sometimes greatly so), methods that can predict response during fluctuating exposures would be particularly valuable. This is especially true for sublethal effects of long-term exposures for which very little is known about the effects of fluctuating exposure.

Parameter error is uncertainty about parameters that have true values, but there are uncertainties in the means by which we measure or estimate those values. This can include random error in a sampling technique or measurement, which might be addressed in part through the use of replication. It can also include situations in which a measurement or parameter estimated is biased, such as might occur if there were an undetected effect of a sample matrix on a chemical analysis. These types of uncertainty are often addressed through quality assurance or quality control procedures, such as the analysis of standard reference materials or matrix spikes. Another approach is to make parallel measurements using different techniques that would not be subject to the same biases.

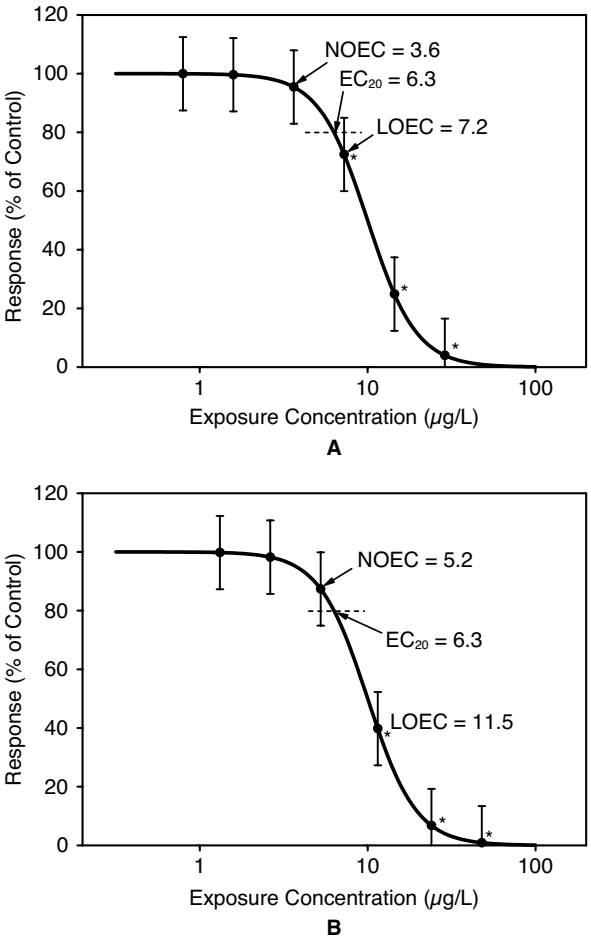

FIGURE 18.6 *Hypothesis testing vs. point estimation*. Results of toxicity tests are generally analyzed in one of two ways: (1) hypothesis testing or (2) point estimation. Hypothesis testing involves statistical testing to determine which treatment means are significantly different from the control treatment or other pertinent reference. Results of this analysis are typically given as the no-observable-effect concentration (NOEC), which is the highest concentration not producing a statistically significant reduction in performance, and the lowest-observable-effect concentration (LOEC), which is the lowest concentration that does cause a statistically significant reduction. Point estimation involves regression analysis of the concentration response curve. The resulting regression is then used to estimate the concentration associated with a particular level of effect; for example, the LC_{50} is often used to express the results of acute toxicity test; it refers to the concentration estimated to cause 50% mortality of the test population at the specified time period (e.g., 96-hour LC_{50}). For sublethal effects (e.g., growth, reproduction), it is common to express point estimates as "effect concentration$_{percent}$" values, such as EC_{20} (the concentration estimated to cause a 20% decrease in performance). Although point estimation has been commonly used for acute test results, hypothesis testing has been widely used historically for chronic test endpoints. There has been a growing movement, however, toward replacing (or at least supplementing) hypothesis testing with point estimation for both acute and chronic data. The reasons are several. For one, despite its name, the NOEC is not necessarily a no-effect concentration; it simply means that no statistically significant reduction was found. The power of a test to detect a reduction varies with the study design and inherent variability of the measure, which is often high for endpoints such as reproduction. This also means that the degree of adverse effect at the NOEC or LOEC varies across studies and endpoints, compromising comparability among tests. In fact, the NOEC and LOEC can vary just as a function of test concentrations. Parts A and B are the theoretical results from two tests showing the same concentration response curve and having equal variability but with different placement of test concentrations. Although the NOEC and LOEC vary between the tests, the EC_{20} values are identical, as they should be because the underlying response was identical. Moreover, the actual ecological impact of toxic effects on a species will be driven by the magnitude of the effect, not by its statistical significance; thus, point estimates provide a better means of comparing results among tests. In addition, regression analyses can be used to develop confidence intervals for point estimates, which allows for quantitative evaluation of uncertainty in risk analyses. For more information on point estimation and its comparison to hypothesis testing, consult Stephan and Rogers (1985), Suter (1996), and Crane and Newman (2000).

Model error is a broad category that might also be thought of as knowledge uncertainty. These are generally cases where the piece of information needed for the risk assessment is not directly measurable and must be inferred or predicted from other information through the use of an actual mathematical model, such as a bioaccumulation model, or in a more conceptual manner. All models have uncertainties, and these uncertainties are therefore made part of the overall uncertainty in the risk assessment when models are incorporated. It is common, for example, for a risk assessment to seek protection of a particular species, but toxicity data for that species may be lacking and require extrapolation from data for other species; assessments involving endangered species are a significant example, as toxicity data are rarely available for endangered species. In this case, knowledge of the toxicology of the chemical can help determine the most effective means of estimating the response of that species to the stressors at hand. In other cases, chemicals of concern may include some for which few or no toxicity data are available. In these cases, tools such as quantitative structure–activity relationships (QSARs) may be useful to estimating the potency of a chemical or predicting species that may be at greatest risk, based on data for other chemicals. The entire issue of extrapolation—among species, among chemicals, among endpoints, among doses—is a major source of uncertainty in risk assessment and one where mechanistic toxicology can be brought to bear most directly.

Another type of model error could be the conceptual uncertainty that results when the processes involved in producing risk are incompletely understood. As an example, there is currently great uncertainty regarding the degree to which exposure of fish to metals in the diet contributes to toxicity, how to quantify that risk, and how to integrate it with risk from concurrent waterborne exposure (Meyer et al., 2005). Another example is how levels of effect estimated from laboratory tests (e.g., 20% reduction in growth) will affect populations of the same species in the field. Tremendous accuracy in determining an LC_{50} will be of relatively little help in most risk assessments if the means to relate that result to the actual populations at risk are lacking. Also, risk assessments often focus on direct effects of chemical exposure (e.g., direct mortality of toxicologically sensitive organisms) to the exclusion of indirect effects (e.g., loss of important food resources), which are often more difficult to predict.

Model error is generally the most difficult type of uncertainty to address in a risk assessment. One way in which model error can be evaluated is through the accuracy of predictions of the same model in other situations where the actual outcomes are known. Another approach is to apply more than one model to the problem and evaluate the concordance of the predictions. In some regulatory programs, generic assessment factors are used, such as dividing an LC_{50} by 1000 to account for unknown or unmeasured chronic effects.

It is common for risk assessors to address uncertainty by choosing environmentally conservative values (i.e., those least likely to underestimate risk) to derive a worst-case scenario; for example, one might choose the most sensitive toxicity test endpoint and compare it to the highest reported exposure concentration. To some degree this approach skirts the uncertainty issue, but it is often appealing because it is expected to place the majority of the uncertainty to one side of the risk prediction by erring toward the side of protection of the resource being assessed. This approach has utility for screening-level assessments—in essence, if the worst case does not show risk, then no more analysis is necessary.

There is a danger in this approach, as well. Risk assessments often combine results of multiple analyses to characterize risk, and the uncertainties are often multiplied in the process. By combining multiple assumptions that are all individually worst-case conditions, one can create an assessment that exaggerates the actual risk. This is easy to imagine in terms of simple probabilities. If a 95% confidence level is chosen as a worst case for a variable, then there is only a 5% chance that the true value is actually that bad or worse. If three conditions are necessary to produce risk, and the value for each of these conditions is chosen with a 95% confidence level (and the distributions are independent), then the probability that all three worst-case conditions are exceeded is $(0.05)^3$, or 0.000125, which is likely beyond a reasonable worst case in most instances.

One way to avoid this stacking of conservative assumptions is through the use of statistical simulations. A commonly used approach is Monte Carlo analysis, a statistical simulation process that estimates the likelihood of various outcomes based on variability in the input variables (e.g., exposure concentrations, species sensitivity distributions). In this approach, each input variable to the risk calculation is described not by a single value but by a statistical distribution (normal, log-normal, Poisson, etc.). For the analysis,

the value for each input variable is selected randomly from the applicable distribution, then risk under that set of assumptions is calculated. This process is repeated thousands of times to generate a probability distribution of expected risk. In general, risk predicted at the 95% confidence limit by this approach will be lower than that predicted by simply making worst-case assumptions at each step along the way; this is because the Monte Carlo simulation recognizes that even if the true value of one of the inputs is near the worst case, it is unlikely that all inputs will be. For this reason, many risk assessors believe that simulations such as Monte Carlo analysis give a much more realistic representation of risk. Such simulations can also be used to evaluate the sensitivity of the risk assessment to various input parameters; for example, one could decrease the uncertainty (variability) of one of the input parameters by some margin, rerun the analysis, and determine what effect that has on the predicted risk distribution. This can help a risk assessor determine whether collecting more data to better define a distribution is likely to improve uncertainty in the overall risk assessment enough to warrant the additional work.

Simulation techniques are quite valuable in exploring the uncertainty in risk predictions and assessing the relative sensitivity of risk predictions to different components of the risk calculation. Nonetheless, one must guard against a false sense of accuracy that could be inferred from the elaborate projections that come from these analyses. Many uncertainties in risk assessment are difficult to describe in terms of sampling distributions (matrix effects on bioavailability of chemicals, uncertainty in lab-to-field extrapolation) or may not even be a part of the simulation model at all (e.g., knowledge gaps).

Challenges for Toxicology to Advance Ecological Risk Assessment

The nomenclature, techniques, and applications of ecological risk assessment have advanced rapidly in the past two decades, but significant hurdles must yet be overcome. The following text explores several such areas that offer challenges to ecotoxicologists as we move forward.

Describing Ranges of Effect Rather Than Single Thresholds

Many risk assessments use specific benchmarks to distinguish between acceptable and unacceptable. As described previously, risk is then expressed as a hazard quotient (ratio of exposure concentration to benchmark concentration) or, if the exposure distribution is known, as the probability or percentage of time that the benchmark will be exceeded (see panel A in Figure 18.4). In either case, one can make statements about whether risk exists, but not about the specific biological effects that can be expected. In other words, what are the expected biological differences between two systems, one with a benchmark exceeded 5% of the time and the other with exceedances 25% of the time? Further, the magnitude of exceedance is not considered, yet clearly this magnitude will alter the type of biological effects that can be expected from the exposure. Where the costs of mitigative measures (e.g., wastewater treatment, contaminated sediment removal) are not excessive, it may be possible to ignore these shortcomings and simply manage exposures such that thresholds for effects are essentially never exceeded; however, when expenditures are large (or even prohibitive), a more refined understanding of expected effects is necessary to properly evaluate cost/benefit for different management alternatives.

Interpreting Sublethal Effects

As discussed above, ecological risk assessments typically focus on measures of effect that can be explicitly linked to changes in populations or communities. Many additional measures (e.g., histological, physiological, behavioral) may be associated with changes in populations when present at sufficient intensity but may not be at lower intensity, and these relationships are not typically understood quantitatively. Many toxicants, for example, induce cellular changes in tissues at exposure concentrations well below those that cause measurable changes in toxicity tests measuring survival, growth, or reproduction, making the ecological significance unclear. Moreover, at low intensities, these measures may actually be part of mitigating against higher level effects. Enhancing our ability to quantitatively use these additional endpoints in ecological risk assessment is a key need.

Identifying Modes of Action Not Represented by Standard Assays

For a variety of reasons, environmental toxicology has come to rely heavily on data generated according to a limited set of standardized test procedures. Standardization of test procedures is very important in the context of providing comparability in data and thereby allowing the development of extrapolation models. Logistical considerations also limit the routine collection of certain types of toxicity data (e.g., life-cycle chronic tests, including reproduction). One consequence of this approach is that standard toxicity tests are not well suited to detect certain types of toxicological effects. For example, the issue of endocrine-disrupting chemicals caught the world largely by surprise and spawned intensive research on the endocrine system. It could be argued that one of the reasons this issue did not come to light earlier is that reproductive and second generation endpoints are rarely a part of aquatic toxicity testing commonly performed on chemicals. The point here is not to suggest that massive testing of reproductive endpoints should be undertaken but rather to underscore the importance of considering modes of action that might lie outside standard testing protocols and developing toxicological tools (such as receptor binding assays in the case of steroid hormone mimics) that can help identify chemicals that may act through these alternative modes of action and thereby be incompletely assessed by common toxicity test protocols.

Better Extrapolation/Reducing Animal Usage

Refining our ability to accurately predict ecological risk puts immediate pressure on having more extensive toxicological data, but both resource limitations and the desire to reduce reliance on animal testing provide pressure to pursue less toxicity testing. To meet both desires, greater ability to extrapolate toxicological data is needed: extrapolation among species, among chemicals, and among endpoints. Current USEPA ambient water quality criteria for the protection of aquatic life require acute toxicity data for at least eight species and chronic toxicity data for at least two of those species (Stephan et al., 1985). Although these requirements were instituted to reduce uncertainty, they also limit the number of chemicals for which criteria can be developed. Because many more chemicals require assessment than have this level of toxicity testing, methods are needed to develop risk estimates based on fewer data, and the means to make this extrapolation without introducing intolerable uncertainty are needed.

Multiple Stressors/Nonchemical Stressors

Although most toxicological data and, for that matter, most ecological risk assessments focus on individual chemicals, real-world exposures are to multiple stressors, both chemical and nonchemical (e.g., nutrients, habitat degradation, temperature). For some of the more ubiquitous chemical mixtures, toxicological models have been developed to assess the potency of aggregate mixtures, such as for polycyclic aromatic hydrocarbons (PAHs) (Di Toro et al., 2001a,b; Swartz et al., 1995), polychlorinated biphenyls (PCBs), dibenzodioxins, and dibenzofurans (Van den Berg et al., 1998). Even more challenging is the development of approaches that integrate the effects of chemical and nonchemical stressors in terms of their combined influence on populations and communities.

Linkage of Organismal and Suborganismal Responses to Population and Community Response

We previously discussed the need to link suborganismal measures to organismal-level responses such as survival, growth, and reproduction. A further challenge is to better link organismal (or suborganismal) responses to population and community level responses; for example, diazinon exposure has been shown to affect chemoreception in salmon, measurably reducing a characteristic antipredation response after an olfactory cue (Scholz et al., 2000). Although it is not difficult to imagine that reduced antipredator behavior could lead to the decline of an exposed natural population, it is also likely that very small changes in behavior would not affect the stability of the population as a whole. What is not clear is how one could quantitatively relate measured behavioral changes to the projected risk to salmon populations.

Conclusion

Environmental management has been an evolving process and the demands on ecological risk assessments have evolved in parallel. In 1966, the Federal Water Pollution Control Administration reported more than 9 million fish mortalities in 372 reported fish kill events in the United States (USDOI, 1967), and this included only reported kills where the extent of the kill was quantified. Given this situation, environmental science of the time focused to a large extent on understanding the causes of these events and establishing thresholds or management practices to avoid such catastrophes. In the face of highly visible fish kills, evaluating the long-term population impact of small reductions in growth probably seemed irrelevant; however, as water quality in the United States has generally improved, increased emphasis has been placed on understanding other less visible (though perhaps no less serious) effects from environmental contaminants. In addition, there is now a heavy emphasis on predicting and avoiding future ecological risks, rather than just reacting to those problems that already exist. On the other hand, overreaction and overprotection in response to potential or perceived risks can create substantial economic and social costs in an increasingly competitive global economy. The application and improvement of ecological risk assessment as a means to objectively evaluate these issues will undoubtedly be important components of environmental decision making well into the future.

Acknowledgments

The principles of ecological risk assessment have emerged from the work of many people. Although guidance published by the U.S. Environmental Protection Agency was used as a primary source material, we wish to acknowledge the contributions of the many, many people whose efforts are embodied in that and other influential documents. We also thank Drs. Richard S. Bennett and Stephen A. Diamond for their comments on the manuscript and Mary Ann Starus for editorial support. This document has been reviewed in accordance with U.S. EPA policy and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Additional Reading

Copies of several documents pertaining to ecological risk assessment under the USEPA Superfund and Pesticides programs are available at http://www.epa.gov/oswer/riskassessment/tooleco.htm and http://www.epa.gov/pesticides/ecosystem/ecorisk.htm, respectively. Also, examples of actual ecological risk assessments can be viewed at http://www.epa.gov/region8/r8risk/eco.html and at http://www.epa.gov/pesticides/reregistration/status.htm.

Unit IV

Case Studies

19

Mining Impacts on Fish in the Clark Fork River, Montana: A Field Ecotoxicology Case Study

Samuel N. Luoma, Johnnie N. Moore, Aïda Farag, Tracy H. Hillman, Daniel J. Cain, and Michelle Hornberger

CONTENTS

Introduction

Large-scale mining and smelting operations are required to satisfy the many demands of modern societies for metals, but few individual human activities visually disturb the Earth's surface more dramatically or have more potential to create hazardous waste problems than a mine. Mining activities cover about 240,000 km^2 of the Earth's surface (Salomons and Forstner, 1984), an area about the size of Oregon in the United States. Understanding such disturbances is essential to remediation of historic legacies and to sustaining responsible mining in the decades and century ahead. In this chapter, we consider the factors involved in determining contamination risks to fish in a mine-impacted river, the Clark Fork River in Montana. Traditional approaches to evaluating ecological risks from mining and metal contamination rely largely on toxicology: comparing controlled studies of metal toxicity to fish with observations of ambient concentrations. In a natural water body such as the Clark Fork, however, risks develop from complex interactions among hydrologic, geochemical, and biological processes that affect both exposure and ecological effects. Both field observations and laboratory experiments are essential to unraveling the complexities of these risks.

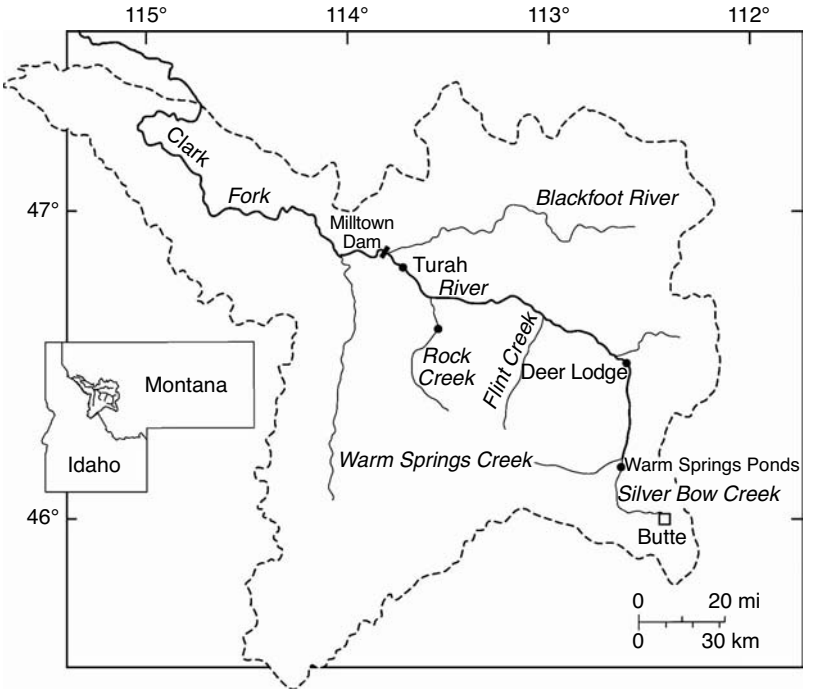

FIGURE 19.1 Watershed of the Clark Fork River in Montana to the confluence of the Flathead River (not shown). The Upper Clark Fork, upstream of Milltown Reservoir, is the subject of this chapter.

History of the Clark Fork Mining and Smelting Complex

In 1805, Meriwether Lewis and William Clark began exploration of what is now Montana. They described the basin of the Clark Fork of the Columbia River as "a unique landscape of primitive beauty" filled with vast resources (Moore and Luoma, 1990). Extraction of these resources began in 1864, with placer mining for gold. By 1896, over 4500 tonnes of copper ore per day was being mined and smelted near Butte in the headwaters of the Clark Fork (Figure 19.1). At the turn of the century, one of the world's largest smelting plants was constructed in Anaconda, 40 km west of the mining operations. In 1955, underground mining of high-grade ores in Butte was superceded by large-scale open-pit mining. Underground mining ceased in 1976. Depressed copper prices forced closure of the smelter in 1980, and mining in the largest open pit slowed in 1983. Mining has resumed in recent years in adjacent pits, along with limited underground operations. When the smelter at Anaconda stopped production, over 1 billion tonnes of ore and waste rock had been mined from the district. The Butte district was touted as the "richest hill on Earth" in its prime. The mining and smelting operations that produced this vast wealth left behind deposits of waste covering an area one fifth the size of Rhode Island. The waste complex comprises the largest Superfund hazardous waste site in the United States (Moore and Luoma, 1990). The adverse environmental impacts found in the Clark Fork basin are typical of the legacy of historic large-scale mining and smelting operations that occur all over the world. Large-scale mining creates visually obvious impacts on surrounding landscapes and watersheds. Most macrofaunal life in a stream can be literally exterminated over relatively small extremely contaminated areas, but less visible effects are more common and more widespread. Evaluating the ecological effects of mine wastes over the broader area, especially, is complicated. It can also be contentious when the stakes are high. In this chapter, we address factors that influence the risks from mine wastes, emphasizing the effects of metal contaminants.

The Mining/Smelting Sites

The specific contamination problems resulting from mineral extraction are ultimately determined by the nature of the ore and what is done to extract its metals. The ore body in the Clark Fork basin consists of high-grade metal sulfide veins enclosed in lower grade rock. The mineral forms of several metals were mined over the history of the deposit. Silver mining dominated for a brief period after the gold was exhausted; zinc was extracted at times, and modern operations remove molybdenum. But, most of the history of the Butte mine involves the extraction of copper. The richest veins of ore (depleted long ago) contained up to 80% copper minerals; the lower grade altered rock around the central ore deposit contains approximately 0.2% copper. The mining activity in Butte removed approximately 400 million m^3 of rock from the subsurface, 90% of which was discarded as waste rock and tailings. Trace element contaminants in the various wastes included arsenic, antimony, cadmium, lead, and zinc. Copper concentrations in discarded mine tailings average 6730 µg/g; in smelter flue residues, they can be 37,100 µg/g (Moore and Luoma, 1990). As a comparison, copper concentrations are 20 µg/g soil dry weight (dw) in unmineralized soils in Western Montana. As much as 35 km^2 of land has been buried under piles or ponds of contaminated materials in the direct vicinity of the mine and smelter. The sulfur content of the Butte ore exceeded 30%, and sulfur accounts for 0.5 to 4.0% of the rock enclosing the ore. Sulfide ores become unstable when they are mined and moved to the oxygen-rich surface environment. Sulfide minerals in the mining waste oxidize, forming acids that leach metals to surfacewater and groundwater systems. Although low pH is common within the pore waters of tailings deposits (Benner et al., 1995), overland acid mine drainage is now rare in the Clark Fork basin. Most of the large-scale contamination is from dispersal of the particulate mine wastes.

The Clark Fork River System

Physical characteristics of a river system influence its exposure to and effects of contamination. Physical processes are a primary determinant of biological characteristics. The size of the watershed and the nature of the tributaries determine the basic geochemical characteristics of the water body and downstream dilution of wastes. Flows, floods, and impoundments affect the distribution of wastes. Other stressors in the watershed can influence ecological impacts. The waste complex in the Clark Fork basin begins in the headwaters of Silverbow Creek at the city of Butte (Figure 19.1). The creek extends approximately 40 km to Anaconda, where the smelter was located. The Warm Springs tailings ponds, adjacent to Anaconda, capture the entire flow of Silverbow Creek at its downstream boundary. The creek is treated with lime, and sediments are settled out in the ponds. Below the release point of the ponds, two other creeks join Silverbow, formally defining the headwaters of the Clark Fork River.

The watershed of the Clark Fork is large, encompassing 57,400 km^2. It is sparsely populated and mountainous, and the major human development is agriculture. Small nutrient inputs from local cities and summer dewatering of the river for irrigation are potential stressors (typical of most Montana streams). In general, the river is a steep-gradient, cobble- and gravel-bottom stream. The Upper Clark Fork River extends 150 km, from below Warm Springs Ponds to Milltown Reservoir (Figure 19.1). It has four major tributaries, each of which approximately doubles its discharge. It has wide floodplains through the uppermost 60 km (the Deer Lodge Valley, extending past Deer Lodge). Downstream from there it is often confined by canyons, with occasional areas of relatively extensive floodplain. Three major river systems join the Clark Fork through this reach. The Lower Clark Fork lies below the confluence of the Blackfoot River and extends to Lake Pend de Oreille. Three additional impoundments and one large pulp and paper mill lie between Milltown Reservoir and the mouth of the Clark Fork. The impoundments have greatly changed the physical nature of the river, and the mill adds waste. These additional stressors add difficulty to evaluating the causes of reduced fisheries in this reach. This chapter considers only the Upper Clark Fork.

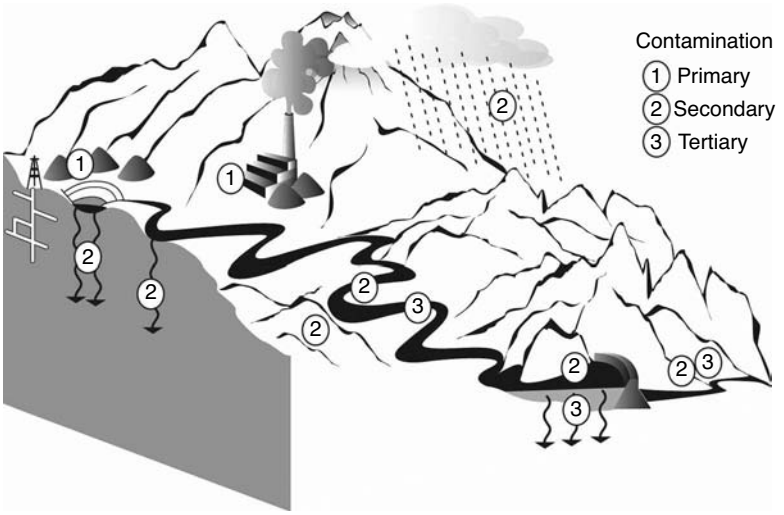

FIGURE 19.2 Primary, secondary, and tertiary contamination associated with mining and smelting activities like those in the watershed of the Clark Fork River. (Adapted from Moore, J.N. and Luoma, S.N., *Environ. Sci. Technol.*, 24, 1279–1285, 1990.)

Geochemically, the upper and lower river waters are near neutral (pH 6.4 to 8.8), and hardness varies from 75 to 350 mg/L. Geochemical characteristics, sediment loads, and stream discharge vary seasonally and between years, as in most rivers of the semi-arid western United States. Mean daily stream discharge on Silverbow Creek near Butte is 4 to 10 m^3/sec. The channel width at low flow is about 3 to 5 m. When the Clark Fork enters Lake Pend de Oreille, 550 km from its origin, mean daily flow in an average year is 634 m^3/sec, and it is the largest river leaving the State of Montana. Although the discharge of Silverbow Creek is only 0.4% of the total discharge of the Clark Fork, enough metal contamination was transported out of its watershed to contaminate the sediments throughout this massive river system.

Distribution of Contamination (Indicators of Exposure)

Dispersal of Contamination from Mining and Smelting

Mining and smelting create primary, secondary, and tertiary contamination (Figure 19.2). Primary contamination is usually spread in a patchwork of tailings and waste rock deposits over the countryside nearest the centers of mining and smelting activity. A pit lake at Butte was created when open-pit mining operations were discontinued and dewatering of the mine was halted (Castro and Moore, 2000). Extensive tailings deposits are confined above the headwaters and in ponds around Anaconda, with associated groundwater contamination. Soil contamination is sufficiently widespread that dissolved and particulate loads of copper increase 100-fold in storm drains during runoff events (Gammon et al., 2005). Smelting at Anaconda also created air pollution, flue dust, and slag. Transport of the waste and contamination away from the mining and smelting sites in streams or through the atmosphere generates secondary contamination in soils, groundwater, and the rivers (Figure 19.2). If they leave the site, contaminated sediments, soils, and their associated metals can be remobilized over and over, continuing their dispersal (tertiary contamination). These processes extend the scale of contamination far beyond the visual disturbances of the landscape.

Effect of the Ore Body

Mineralized zones inherently have high metals concentrations in water and soil (soil anomalies). One of the most contentious questions asked about mining impacts is how much of the contamination was there naturally before the mineral deposit was disturbed and how much is a result of mining activity.

The question is of particular interest with regard to sediments, which are the repository of greatest mass of contamination. Culpability for clean-up often depends on defining which activity was responsible for the sustained contamination of sediments. The primary effect of mining and smelting is to disperse the contamination that occurs naturally in the Earth. The size of the ore body and the concentration of a particular metal in the soils covering the ore body are important determinants of the degree of dispersal. The area of metal-enriched soil around an undisturbed deposit generally ranges from 0.4 to 2.4 times the exposed area of the ore body; for example, an ore body that has a surface expression of 1 km^2 might show a surface anomaly of 0.4 to 2.4 km^2. Mining greatly expands the fingerprint of the ore body on the surface of the Earth, as metal-rich material is dug up and moved around. Differences in the area of the surface anomaly contribute to major differences in the distance that uncontained metals are transported away from mined compared to unmined ore bodies (termed the *dispersion train*). Comparisons of a variety of mines show that mining exaggerates the size of the natural ore body anomaly from about 100 to 500 times the natural anomaly (Helgen and Moore, 1996).

In the Butte mines, the major ore body has a surface area of approximately 4 km^2. After 150 years of mining activity, primary wastes adjacent to the open pit mine cover an area of about 50 km^2. In total, about 1400 km^2 of land is contaminated in the Clark Fork basin by the wastes generated from mining, processing, and smelting at Butte and Anaconda. Comparing after-mining metal concentrations with the before-mining estimate of dispersion shows that approximately 2200 times more metal was released into the upper Clark Fork basin from mining than would be expected from the original mineral deposit. Before mining in the basin, sediment metal concentrations would have reached background concentrations in about 20 to 30 km, based on cumulative basin area modeling (Helgen and Moore, 1996). Now, after mining, contaminated sediments are dispersed into Lake Pend de Oreille, approximately 600 km downriver from the mine (Axtmann and Luoma, 1991). Atmospheric deposition has also contributed to metal contamination across the watershed. Between 1900 and 1980, the Anaconda smelter processed much of the locally mined Butte ore, with a dispersal range of up to 300 km^2 (Moore and Luoma, 1990). Arsenic, cadmium, copper, lead, and zinc associated with the smelting process caused adverse impacts to soils, cropland, and farm animals.

Silverbow Creek

It is estimated that 2 million m^3 of tailings was dumped directly into Silverbow Creek. The massive addition of sediment clogged the original stream and increased the potential for flooding (Weed, 1912). Floods, accentuated by the aggraded channels, deposited that material onto floodplains all along Silverbow Creek and the Upper Clark Fork (Brooks and Moore, 1989). As a result, unvegetated, low-pH tailings deposits (termed *slickens*) lined Silverbow Creek over its entire length, with occasional patches of metal-tolerant grasses and willows. The slickens contained contaminated soil up to 2 m thick; some of the deposits were over 200 m wide. In 2002, typical metal concentrations in fine-grained sediments (within 5 km of the inlet to the ponds) were cadmium, 21 μg/g dw; copper, 1660 μg/g dw; zinc, 5690 μg/g dw (Dodge et al., 2003). Premining concentrations, determined from soil pits, were cadmium, <1 μg/g dw; copper, 16 μg/g dw; zinc, 49 μg/g dw (Ramsey et al., 2005). The lack of vegetation in the slickens also increased the erosion potential of the banks and adjacent floodplain, resulting in continual recontamination of the stream and aiding downstream transport of the wastes. Everman (1892) found the waters of Silverbow Creek to be "the consistency of thick soup, made so by the tailings it receives from the mills at Butte. No fish could live in such a mixture." Invertebrates were absent over the entire 40 km of Silverbow Creek before 1975, after which the earliest treatments of mine wastes began to take effect and metal-tolerant species began to appear (Chadwick et al., 1986). No fish were found in the stream in published surveys through 2000 (see later discussion).

It is increasingly recognized that environmental remediation is a necessity as ore bodies are depleted and mines or smelters are closed, but remediation can be a challenge where the extent of the problem is large or the waste problem is complex (Moore and Luoma, 1990). Remediation efforts were initiated on Silverbow Creek in 1988 as part of the USEPA's Superfund cleanup process. Early efforts removed contaminated soils or covered areas of the most severe contamination around the smelter, but the first 10 years were mostly dominated by litigation-driven conflict and proprietary data collection linked to

the litigation. In 1998, the State of Montana was awarded $260 million to clean up the primary waste deposits in the 40-km reach of Silverbow Creek between the mine and the smelter. In the early remediation, tailings were removed, and streambeds were reconstructed from experimental segments of the creek. By 2005, a massive effort was underway to reconstruct the creekbed over large areas and to move all the major tailings deposits from the Silverbow floodplain to the large, historic tailings ponds near Anaconda. The slickens were replaced with uncontaminated fill. Today, metal-tolerant grasses are becoming established over many of the former slickens, changing both the appearance and the ecology of the riparian zone. Anecdotal evidence indicates that invertebrate communities are gradually increasing in diversity and abundance in the creek, and anecdotal reports of fish in the stream have also appeared in recent years. It is not yet clear whether or not tailings left behind under some of the fill will cause problems in the future, and there are questions about the stability of the remediated creekbed during floods. Most important, however, systematic and detailed studies documenting successes and failures are not yet a part of the remediation effort. Nevertheless, improvements are obvious as a result of one of largest remediation efforts in any mining region in the world. Limits to progress reflect the massive extent and complexity of the problems, and the short period of the restoration effort (a decade) compared to the 15 decades of damaging activity before it began.

Warm Springs Ponds

Modern, well-run mining operations capture and contain tailings in (sometimes extremely large) pond systems, although well-known exceptions also exist (e.g., the OK Tedi mine in Papua New Guinea is built on extremely steep terrain and releases tailings directly into the Fly River). Between the 1870s and 1950s, little effort was made to capture contaminated soils, sediments, or waters from the Clark Fork mining and smelting operations. Historically, the Clark Fork River was turbid with sedimentary mine waste for over 200 km downstream, at least periodically into the 1950s. Before the 1900s, Evermann (1892) seined the Clark Fork River near Deer Lodge (Figure 19.1) and found no fish. He stated that the river "is said to have been well supplied with trout and other fish, but none has been seen since the concentrators began operations." In the 1950s, ponds were completed that contained smelter wastes and mine tailings. Another set of ponds attempted to capture Silverbow Creek near its terminus and settle out contaminants. Through the next three decades the pond systems were upgraded, and water treatment (e.g., liming) was added; however, sedimentary wastes bypassed the ponds during high flows until the extensive upgrades were complete in the 1990s. As much as a meter of tailings was removed from stream channels external to the ponds as a part of the remediation efforts in the mid-1990s. Releases of zinc and arsenic increase during high discharges into the ponds, but most anecdotal signs are that the contaminated sediments from Silverbow Creek are now efficiently captured in the pond system. The system has not been tested by a large flood. Presumably, progressive improvement of the containment system (tailings ponds) was a major factor in improvement in biological quality of the Clark Fork River. Some trout began to reappear in the river below Deer Lodge in the late 1950s (Spindler, 1959). Virtually no trout were present immediately below the ponds until 1973, one year after some major improvements in waste treatment capabilities were installed. Since 1973, brown trout (*Salmo trutta*) have been the most abundant trout at the site, but their numbers have fluctuated widely. A few rainbow trout (*Oncorhynchus mykiss*) are found below the ponds, but native trout (bull trout and cutthroat trout) are rarely, if ever, observed.

The Environment within 2 km of the Warm Springs Ponds

The environment in the first 2 km from the Warm Springs Ponds is a unique habitat in the river. It is worth mention because its characteristics have confounded attempts to relate mining activities to contamination and ecological change in the river. Sediments and benthos adjacent to the outlet from Warm Springs Ponds were first sampled for metal contamination in the mid-1990s. Concentrations of copper, cadmium, and zinc were as low as stations subjected to much more dilution downstream (Table 19.1). The waters of Silverbow Creek are treated with lime and progressively settle into an anoxic bottom as they move through the ponding system. This appears to be effective in reducing metal inputs to the stream just below the ponds, at least during low-flow periods of the year, although arsenic inputs have

TABLE 19.1

Copper Concentrations in Fine-Grained (<64 μm) Sediment and the Metal-Tolerant Caddisfly *Hydropsyche* Species from above Warm Springs Ponds, Immediately below the Outfall from the Pond System (–2.1 km), and Sites Further Downstream in the Clark Fork River

Kilometers from Head of Clark Fork River	Copper Concentration (μg/g dw) in *Hydropsyche* Species	Copper Concentration (μg/g dw) in Sediments
Silverbow above ponds	365	1663
–2.1 (Silverbow at the outfall of the Warm Springs Ponds)	31	169
11	122	1650
45	114	1200
85	62	748
190	54	277

increased. Within 10 km, metal contamination reappears as banks begin slumping into the river (Table 19.1). Metal loads in the water of the Clark Fork between 1991 and 1995 showed that erosion of historic mine tailings from highly contaminated banks and floodplains in the approximately 40 km below the pond anomaly was responsible for most of the continuing contamination downstream (Hornberger et al., 1995). Anecdotal evidence suggests that the system of ponds itself is a uniquely productive habitat. This appears to help support a biologically rich habitat just below their outlet. Visibly hardy trout populations, presumably migrants from streams less contaminated than Silverbow Creek, live and grow in the ponds and appear to migrate into the river downstream.

Invertebrates are highly abundant in the stream just below the ponds, although the community lacks metal-sensitive species. It is the only habitat in the upper Clark Fork where amphipods (*Hyalella*) are found. Brown trout in this section, and a few kilometers downstream, are more abundant than anywhere else in the river. From 1973 to 1978, brown trout averaged about 500 trout per mile during spring sampling in this area (Figure 19.3). During the period from 1979 to 1985, the numbers of brown trout nearly tripled, averaging about 1300 trout per mile. Brown trout densities of 1600 to 2400 trout per mile were recorded from 1985 to 1996, but in 1998, only 750 brown trout per mile were found.

In the early 1990s, the first 2 km below the ponds was characterized by extensive willow growth along a shallow riparian zone. Tailings-laden banks did not occur for about 5 km downstream. Remediation in the late 1990s (Figure 19.4) removed the willows and associated soils and replaced them with a relatively broad cobble riparian zone. This removed some metal contamination but also destabilized the bed of the stream. A paucity of studies since 1998 prevents further determination of whether the long-term effects of remediation have been to improve or reduce fish abundance in this small area; nevertheless, a peak of trout abundance remains in this area. The peak confounds correlation of trout densities in the Clark Fork with proximity to mining activities. In the absence of exposure data showing the pond effect, this peak has exacerbated contentious discussions regarding the impact of the mining and smelting activities.

Upper Clark Fork River

Floodplain and Bank Contamination

The modern watershed of the river still has widespread slickens through its first 60 km (in the Deer Lodge Valley). Cut banks containing a meter of contaminated fine sediments occur all along the upper 308 km of the river. These thick banks of fine-grained sediments typically are contaminated with metals characteristic of mine wastes (Axtmann and Luoma, 1991; Moore et al., 1989). Their lead isotope ratios match those of the mine, rather than the watershed (R. Bouse, USGS, unpublished data). In the upper 60 km of the Clark Fork River, copper concentrations are 1000 times greater than is typical in the banks of tributaries. Banks closer to Milltown Reservoir are visually similar to topsoil, but copper concentrations can be 20 to 25 times higher than is typical of uncontaminated tributaries. Thus, every time the upper river meanders and undercuts a bank, sediments that contain substantial concentrations of metal are reintroduced to the river by the slumping bank. The distributed nature of the contamination in the

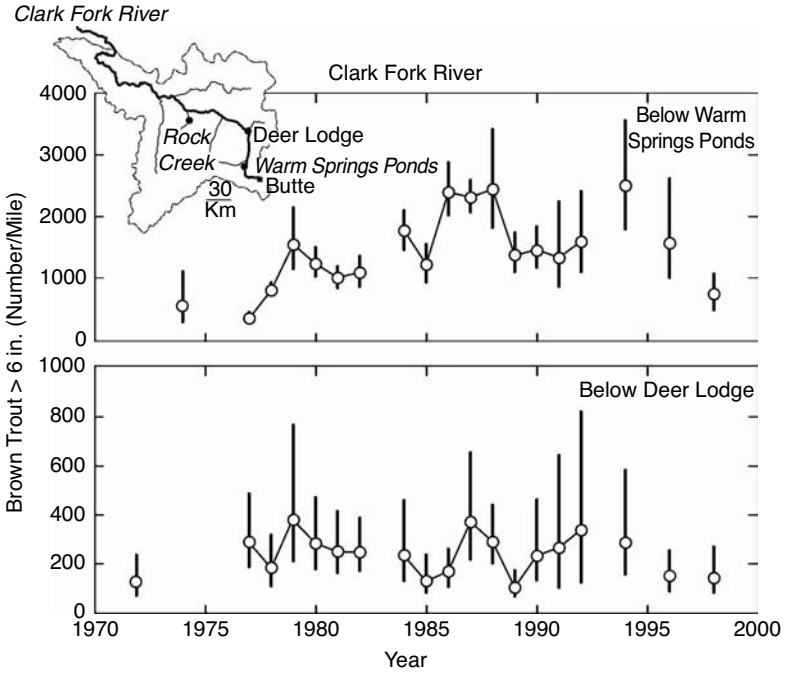

FIGURE 19.3 Fluctuations in the abundance of brown trout (*Salmo trutta*) in the trout-rich, low-contamination habitat downstream from the Warm Springs Ponds and in recontaminated habitat from near Deer Lodge. Note the difference in scale on the *y*-axes.

floodplains of the Clark Fork River also presents a remediation problem of massive scope and complexity. Natural processes will eventually replace the floodplain sediments of a meandering river, over thousands of years. Humans could physically remove the millions of tonnes of sediments in such a floodplain, but important questions about where to put the waste must first be resolved. It is also possible that removal of the floodplain sediments would destabilize the river bed and could result in destroying as much or more fish habitat as does the toxicity of sediments.

Water Contamination

Acute episodes of metal input to Clark Fork waters, accompanied by massive fish kills, once occurred with regularity. The soils of the slickens contain high concentrations of precipitated metal salts (Nimick and Moore, 1991). When mixed with rain water, the precipitates readily dissolve, generating acidic pools as well as groundwater inflows and surface runoff that contain trace element concentrations thousands of times higher than uncontaminated natural waters. Intense rainstorms in summer have washed large amounts of this dissolved metal and acidic water into the river in the past. The river would become acidic, red with iron mobilized from the floodplain, and enriched with extreme metal contamination (Averett, 1961; Lipton, 1993). Throughout the 1960s and early 1970s, anecdotal reports cited dead fish during the periods of red water and elevated metals concentrations (Peters, 1975). Eight kills were documented between 1983 and 1992 following thunderstorms (Averrett, 1961; Johnson and Schmidt, 1988; Phillips and Lipton, 1995). Numbers of dead fish ranged from less than 20 near Rock Creek in 1959 to more than 10,000 from the Mill-Willow Bypass to Racetrack in 1984. One of the early remediation actions in the early 1990s was construction of berms (dikes) on slickens adjacent to the river. The berms successfully prevent overland flow of rainwater into the stream, as long as they are maintained. Observations of discolored river water and large numbers of dead fish essentially disappeared after the mid-1990s.

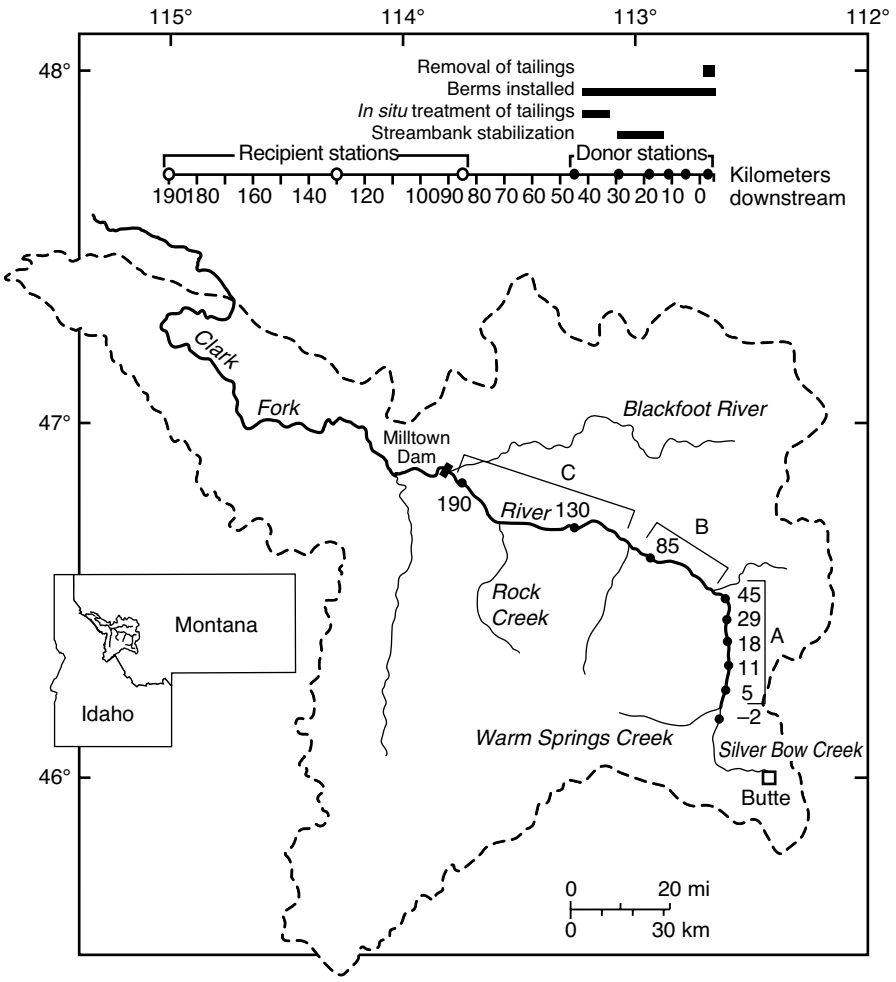

FIGURE 19.4 Map locating different remediation activities in the floodplain of the Clark Fork River below Warm Springs tailings ponds. Black bars indicate the reach of the upstream river along which different remediation activities took place. The upstream stations are the primary source of contamination (donor stations) for the downstream stations (recipient stations).

Small episodes of toxicity could still occur from pulses of inflow from the floodplain, but they are difficult to document. Typically, dissolved concentrations from a water body are compared to laboratory-derived measures of toxicity to determine if organisms (fish) are at risk for metal toxicity. For example, the U.S. Environmental Protection Agency (USEPA) defined hazard quotients (HQs) for different localities in the Clark Fork River from the ratio of "site exposure levels" (dissolved metal concentrations) to "levels believed to cause no or minimal effects" based on the dissolved toxicity database for trout (USEPA, 1999). From toxicity testing data, the USEPA concluded that acute lethality to trout occurs at dissolved copper concentrations greater than 30 to 40 μg/L (USEPA, 1999). The chronic effects of a dissolved metals mixture was studied in the experiments specific to Clark Fork conditions (Woodward et al., 1994, 1995). They verified that concentrations of copper, cadmium, and lead at the water quality criteria resulted in chronic effects, manifested as decreased growth of brown trout fry at 26 through 88 days. The limits of toxicity testing for defining the well-being of fish are well known (Luoma, 1995). Less appreciated are the challenges of identifying the ambient exposure in the water body.

The most widely collected dissolved metal data for the Clark Fork River are the quarterly analyses from water quality monitoring stations (Dodge et al., 2003). Such infrequent sampling is insufficient to

characterize the types of variability typical of mine-impacted streams; for example, the hyporheic zones under the bed of a contaminated stream or in the subsurface of a slicken deposit are microbially and geochemically heterogeneous environments (Wielinga et al., 1999) that contain acidic, metal-rich pore water (Benner et al., 1995). Snowmelt, early spring flushing events, or post-rain surges can transport these metals to the stream (Nagorski et al., 2003). Such variability in metal concentration occurs on weekly, monthly, or seasonal scales. Concentrations in a contaminated stream also fluctuate regularly during the day (Nagorski et al., 2003) and can be two- to threefold higher at night than during the day in the Clark Fork (Brick and Moore, 1996). The short-term variations may be as great as the longer term variability, and both may have important implication for toxicity.

Dissolved copper concentrations in the quarterly data for the Clark Fork River ranged from 2 to 20, with only 2 of 232 observations exceeding the acutely lethal concentration. Chronic dissolved exposures to metals also did not suggest effects at the average concentrations (Woodward et al., 1994, 1995); however, survival of trout fry and fingerlings placed in cages in the Clark Fork River for several months was lower than for reference streams in the late 1980s (Phillips and Lipton, 1995). The USEPA concluded that acute toxicity rarely if ever occurred in the river, but the Agency could not explain either the caged fish mortality or the reduced fish populations of the river. Although large overland inputs of contaminated water were not observed during this period, less visible acute pulses of metal input were suspected, from groundwaters or the hyporheic zone (USEPA, 1999). The overall conclusion was that survival was affected by "exposures to pulses, or other high concentration events," although none was documented. They also suggested, but could not quantify, a role for nonmetal stressors in affecting fish. Both conclusions were debatable, given their weak direct documentation, but the conjecture about pulse inputs was consistent with the complex behavior and challenges of sampling contamination in the water column of a mine-impacted river.

Sediment Contamination

When contaminated particulate or sedimentary material is dispersed through an ecosystem, it equilibrates with water, detritus, and living food materials, resulting in ongoing contamination of all environmental compartments. Organisms (including fish) are exposed to this milieu throughout their lifetime. This type of chronic sediment contamination may reduce or eliminate populations of fish without killing adults (e.g., by inhibiting reproduction) or without leaving visually clear evidence of effects. Chronic sediment contamination can also be widespread. The occurrence, distributions, and geochemistry of the dispersed material, as well as ecological characteristics, determine biological exposures to contamination.

The heterogeneous mix of mine-derived materials in a river bed usually includes natural silt-clays, sand, gravel, or cobble. When metal concentrations are compared among samples with such different particle-size distributions, the results can be very difficult to interpret. The highest concentrations of metals typically associate with fine-grained sediments. Sediments dominated by sand typically have lower metal concentrations because of a smaller ratio of surface area to mass. Fine-grained sediments are also remobilized easily and are most relevant to biological exposures. Separation of the fine fraction of sediments for analysis reduces the physical variability among sediment samples, and that reduces the impact of grain size variability on concentrations (Salomons and Forstner, 1984). Interpretation of much of the early metal data from sediments of the Clark Fork River was highly confounded by grain-size variability. Characterizations of metal contamination from concentrations in fine-grained, sieved sediments (<64 μm) resolved that variability and diffused some of the contentious discussions over contamination trends in the river (Axtmann and Luoma, 1991; Brook and Moore, 1988). In a mine-impacted river, local precipitation events and subsurface inputs can confound the typical particle size relationship (Moore et al., 1989), especially in the most contaminated areas. Sieved sediments are still effective measures of concentration in such areas but should not be used to evaluate metal loads in the system.

When particle size biases are eliminated, the primary determinants of downstream trends in sediment contamination away from a mine site include the size of tributaries, their sediment load, and their buffering. As the sediment moves downstream away from the mineralized zone, it mixes with sediment from tributaries draining uncontaminated surrounding areas. The unenriched sediments dilute the

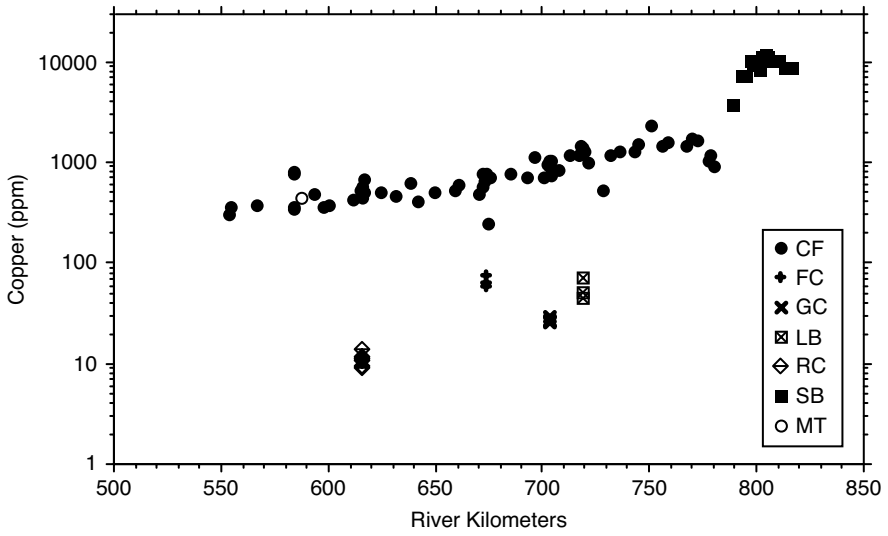

FIGURE 19.5 Exponential decline in copper concentrations (μg/g dry wt) in the <63-μm fraction of sediments, as a function of river mile, from Silverbow Creek (SB), tributaries of the Clark Fork (RC, FC, GC, LB), the Clark Fork (CF), and Milltown Reservoir (MT) (Moore et al., unpublished data). Note the persistent high concentrations in Silverbow Creek, the low copper concentrations in tributaries, and the exponential decline of contamination along the Clark Fork River. Despite dilution from major tributaries, copper concentrations in the Clark Fork sediments are ≥10× higher than concentrations in tributary sediments over the entire 250 km of river.

concentration of metals in the stream sediment at every tributary juncture. A simplified estimate of the amount of sediment supplied from a particular drainage can be obtained from the cumulative area of the basin (Helgen and Moore, 1996), as long as the climate and geology are similar.

On a scale of 600 km, bed sediment metal concentrations in Clark Fork River decline downstream from below the pond anomaly, following a single logarithmic function (Figure 19.5) (Axtmann and Luoma, 1991; Helgen and Moore, 1996). Copper concentrations in the uppermost Clark Fork River are >100 times higher than in tributaries without a major history of mining. Concentrations of cadmium are 67 times higher. At a site 368 km from the mine, copper concentrations were found to be 10 to 20 times higher than concentrations in tributaries. A strong fit to the cumulative basin area model (Helgen and Moore, 1996) indicates that tributary sediments progressively dilute the upstream source. The banks in the first 50 km of the river appear to be the likely source (Hornberger et al., 1995). The function describing the decline predicts that the distance necessary to dilute metal concentrations by half was 100 to 180 km; contamination from the mine could extend 475 to 750 km in an unimpeded river system.

Although downstream trends were quite distinct in the 600-km spatial scale, distribution of metal contamination was more complex on smaller scales. Axtmann et al. (1997) noted that metal concentrations in the middle reaches of the Upper Clark Fork River slightly exceeded those predicted by watershed dilution of metal contamination. They attributed this to inputs by contaminated local banks. Hydrologic and geomorphologic processes, such as floods, new bank cuts, and variable mobilization of fine-grained deposits within the river bed, also will change local contributions to the gradient, in unpredictable ways. Ice jams in some years and not others can redistribute contamination spatially and affect year-to-year comparability (Moore and Landrigan, 1999). Incomplete mixing of sediments near tributary confluences can reduce metal concentrations in the immediate vicinity, only to have them return to higher values further downstream (this is a common feature around tributaries in the upper Clark Fork River) (Axtmann et al., 1997). Monthly metal (copper) concentrations in fine-grained sediments show that as much as twofold variability in sediment-metal concentration is common within a site, following seasonal patterns in some cases (Figure 19.6). With less frequent data, it is difficult to differentiate metal concentrations between two stations as far apart as 50 km in the upper Clark Fork (Axtmann and Luoma, 1991).

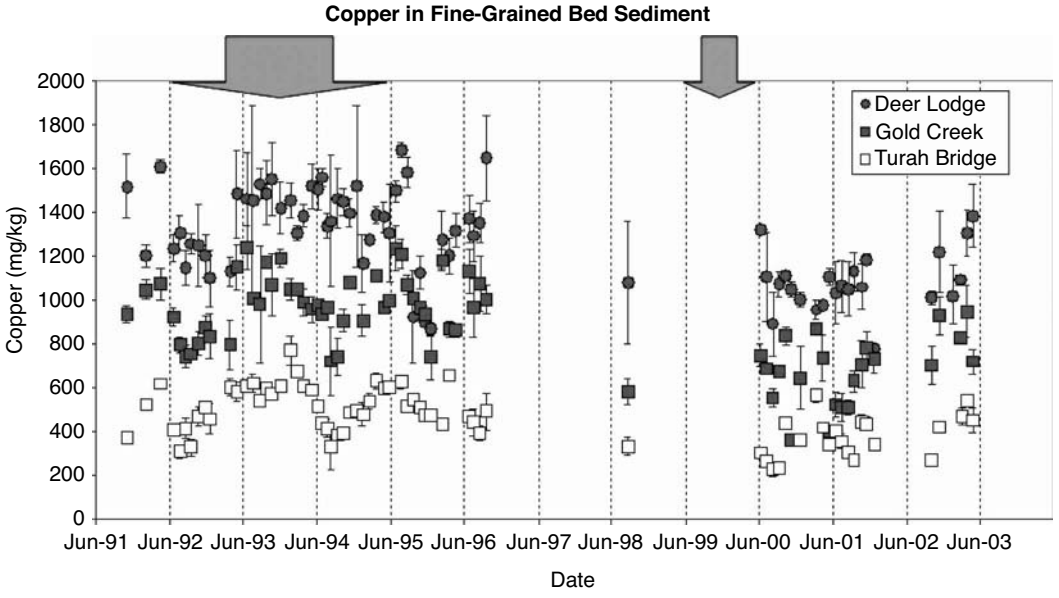

FIGURE 19.6 Metal concentrations in the <63-μm fraction of sediments from three stations in the Clark Fork River, collected nearly monthly in most years between 1991 and 2003 (Moore et al., unpublished data). Concentrations fluctuate seasonally, at least at some stations. Concentrations at each station were generally lower from 2001 to 2003 (after remediation) than from 1991 to 1996 (before completion of most of the remediation), but monthly data are necessary to clearly depict the trend.

Remediation activities have extended into the upper 60 km of the Clark Fork basin, below the tailings ponds, with the goal of reducing sediment contamination. Berms were built to reduce overland runoff, selected areas of tailings were removed or treated *in situ*, and banks were stabilized in selected reaches (Figure 19.4). Effects of remediation can be evaluated by comparing the contaminated sediments in the period from 1991 to 1996 with the period from 2000 to 2003 (Figure 19.7). Mean concentrations of copper at all stations were lower in the latter period, but there was great overlap among the data. The major change was that the maximum concentrations and the minimum concentrations had both declined. Closer analysis of a single station indicated that concentrations may have begun to increase in 2002 or 2003, raising the possibility that the effect of the initial clean-up was not being sustained (Figure 19.7). More data are necessary before a final conclusion is possible. The Clark Fork data illustrate that the scale of contamination in a mine-impacted river system can be massive and that small-scale variability can be formidable. Both are important considerations for ecologists seeking "control" sites within the river or choosing study scales for comparisons of community differences within a river.

Reservoirs

Contamination from the Butte and Anaconda mining activities occurs in the sediments of all impoundments along the river. In 1990, the relatively small Milltown Reservoir at the confluence of the Blackfoot River (Figure 19.1) contained approximately 100 tonnes of cadmium, 1600 tonnes of arsenic, and 13,000 tonnes of copper. Public water wells adjacent to the reservoir were closed in the 1980s because of the arsenic contamination. Contamination also occurs in the three reservoirs that lie between Milltown Reservoir and Lake Pend de Oreille, at levels consistent with the downstream dilution that occurs as the watershed progressively dilutes metals from the mining and smelting activities.

Invertebrate Exposures to Contaminants

The dose of metal that an organism experiences in a water body is determined by the bioavailability of the metal at the scale proximate to the organism. Multiple pathways of metal exposure exist in a water

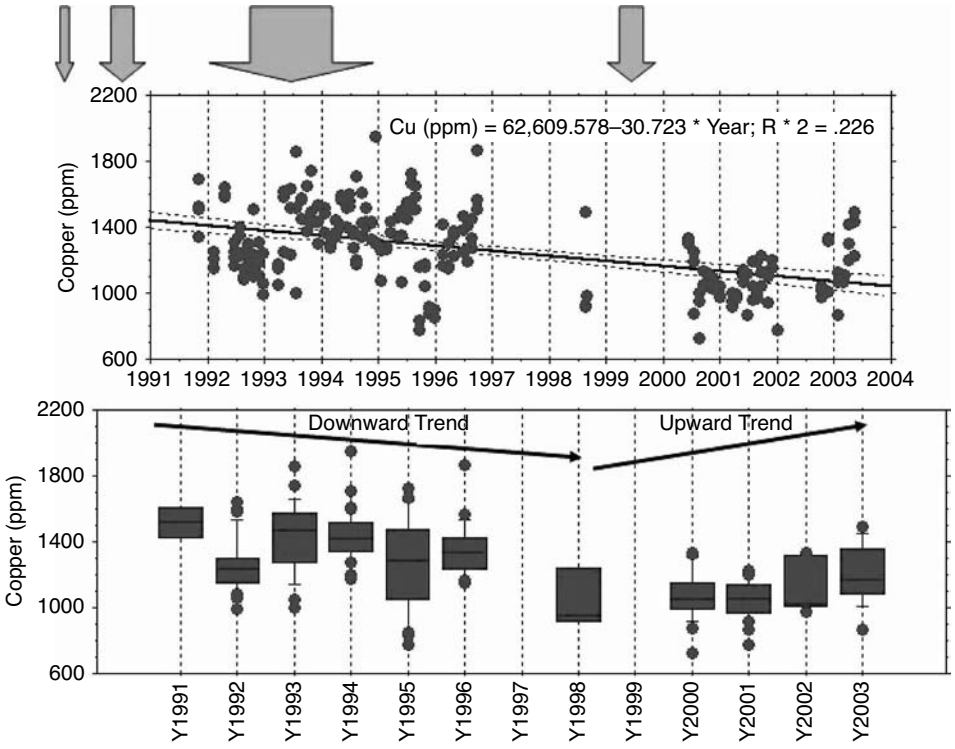

FIGURE 19.7 Fluctuation in and annual mean (with standard deviations and ranges) of copper concentrations at a location near Deer Lodge on the Clark Fork River (Moore et al., unpublished data). Concentrations were at their minimum in 2000 but may have begun to increase after that.

body contaminated by particulate mine wastes (e.g., dissolved metal that passes across the gill and body surface, ingested food and particles ingested with food). Contaminated organisms from lower trophic levels (e.g., plants or detritus and their consumers) are eaten by upper-trophic-level animals (e.g., fish).

Determinations of bioaccumulated metal offer a method for directly evaluating the dose of metal that different species are experiencing. The insect community is one of the most important faunal components in cobble-bottom streams, an important source of food for fish, and an excellent choice as an indicator of bioavailable metal contamination (Cain et al., 1995). Metal concentrations in these invertebrates, therefore, can be used to complement water, sediment, and fish tissue analyses as exposure indicators (Phillips and Rainbow, 1994). One indicator species used in studies of Clark Fork contamination was the caddisfly (*Hydropsyche* sp.) (order Trichoptera) (Cain et al., 1992). These animals are relatively sedentary, widespread in occurrence, and of sufficient mass for analysis. They live for a year or more as aquatic larvae and inhabit riffle zones where they are an important food for local fish.

In general, copper concentrations in the *Hydropsyche* were about 10% of the concentrations in fine-grained sediments in the Clark Fork. Bioavailable metal (both whole-body and cytosolic concentrations in insects) followed the same general gradient as did sediment concentrations of copper and cadmium through the mid-1990s (Figure 19.8). Spatial trends in bioaccumulation seen in the *Hydropsyche* were generally seen in other species as well, although absolute concentrations of copper and cadmium differed widely among species (Cain et al., 1992). All taxa were contaminated relative to reference sites (tributaries). Strong correspondence to other indicators of contamination (e.g., sediments or water) were also evident.

Cain et al. (1995) found that the presence of undigested gut content resulted in approximately 30% higher whole-insect copper concentrations, compared to animals whose digestive tract were removed. The presence of undigested gut content also slightly increased the variability in metal concentrations among samples; nevertheless, undigested gut content did not affect comparisons of contamination among

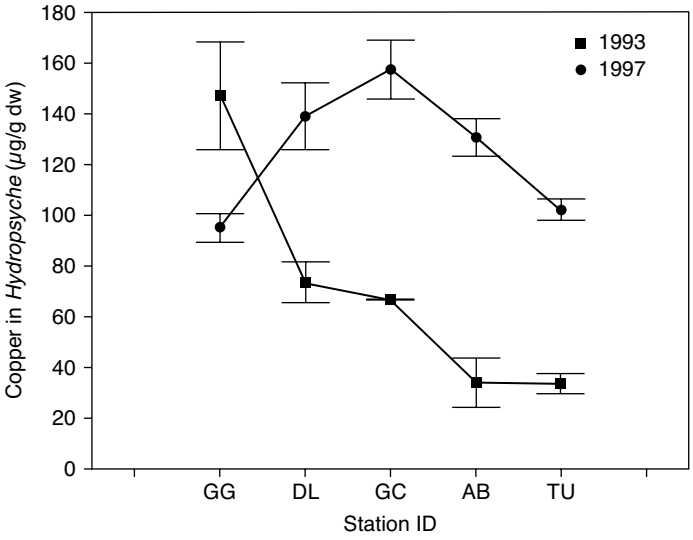

FIGURE 19.8 Copper concentrations in the bioindicator (larvae of the *Hydropsyche* sp.), extending from a recontaminated station below the Warm Springs Ponds (GG) downstream to the Turah station (see Figure 19.1). Before remediation, copper followed the clear exponential gradient downstream typical of sediment contamination. Remediation reduced bioavailable copper concentration more upstream than mid-river, changing the spatial distribution trend.

sites or among species (Cain et al., 1995). Cain and Luoma (1999) avoided the ambiguities of external metal and undigested gut content by analyzing metal concentrations within the cell solution of insects (the cytosolic material inside cells as isolated by homogenization and ultracentrifugation). Cytosolic concentrations of copper in the *Hydropsyche* were approximately 20 to 60% of whole-body concentrations and correlated strongly with whole-body concentrations. This evidence clearly shows that the copper contamination was assimilated into invertebrate tissues and was available to disrupt biochemical processes. The metal in cell solution is probably readily available for trophic transfer to predators, as well (Wallace et al., 2003).

Year-to-year variability in metal contamination was typically greater in insects than in sediments in the Clark Fork, although conclusions were based on only one collection per year. One result was that the effects of remediation efforts were more complex to interpret in insects. A trend of declining concentrations in the *Hydropsyche* was evident in stations from the Deer Lodge Valley, between 1986 and 2003, but year-to-year variability overwhelmed any trends in the reaches of the river downstream from Deer Lodge. Higher concentrations were observed in years of high river discharge than in years of low river discharge (presumably reflecting the greater particulate or dissolved input from runoff in years of greater discharge). Concentrations of copper and cadmium in the insect larvae correlated significantly with annual discharge for all the years at the downstream stations. The result was that the spatial trend in metal concentrations in the *Hydropsyche* shifted to higher concentrations mid-river than in the uppermost river, especially in high flow years (Figure 19.8).

Adverse Effects on Invertebrates

Field observations and *in situ* microcosm studies (Clements, 2004) show that the structure and function of invertebrate communities change in streams subjected to metal contamination from mine wastes but recover when metal contamination is remediated. In some cases, the abundance of benthic macrofauna can be reduced by the contamination, but not always. Insensitive species can be very abundant and show little sign of stress in some contaminated environments (Lefcort et al., 2000). On the other hand, the species richness of the benthic community consistently is reduced in the most contaminated areas, but it is not an especially sensitive measure of effects as contamination declines. *Hydropsyche* were

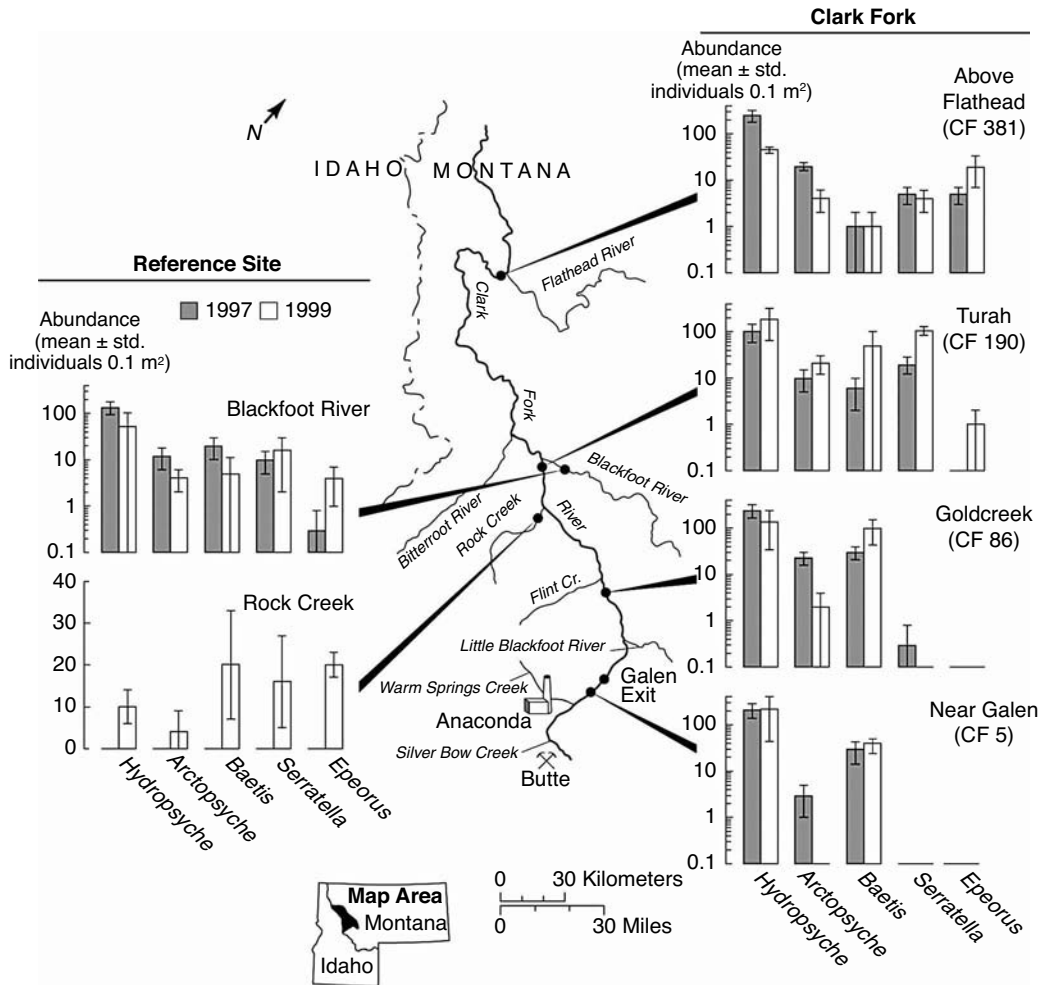

FIGURE 19.9 Abundance of five species of insect larvae at different stations in the Clark Fork Watershed (data from McGuire, 1999; Cain et al., 2004). Heptageniid mayfly species (*Epeorus* and *Serratella* spp.) that were found to be sensitive in other studies (Clements, 2004) were persistently absent at the sites that were most contaminated in the Clark Fork. Of these five species, only *Hydropsyche* sp. are found in Silverbow Creek.

sometimes extremely abundant. Some species from the typically absent orders of mayflies and stoneflies progressively disappeared upstream.

The most sensitive changes in community attributes stem from species-specific differences in sensitivity to metals (Clements, 2004). At a level of contamination where no change in species richness is detectable, sensitive species can be extirpated, survivors physiologically impaired, and ecosystem functions lost. In the Clark Fork watershed, for example, Silverbow Creek was always impaired, with both lower abundances and lower numbers of species. Species richness (and other community metrics) in the Upper Clark Fork was reduced in the Deer Lodge Valley (upper 60 km of the Clark Fork) through the 1990s but appeared to be quite similar throughout the river after that. But, some species of mayflies (Ephemeroptera within the family Heptageniidae) and stoneflies (Plecoptera) are absent in areas of moderate contamination, where changes in overall species richness are not detectable (McGuire, 1999) (Figure 19.9). The widespread abundance of *Hydropsyche* (Trichoptera) across the contamination gradient in the Clark Fork River is also consistent with a body of literature showing that oligochaetes, chironomids, and hydropsychid caddisflies are relatively metal tolerant.

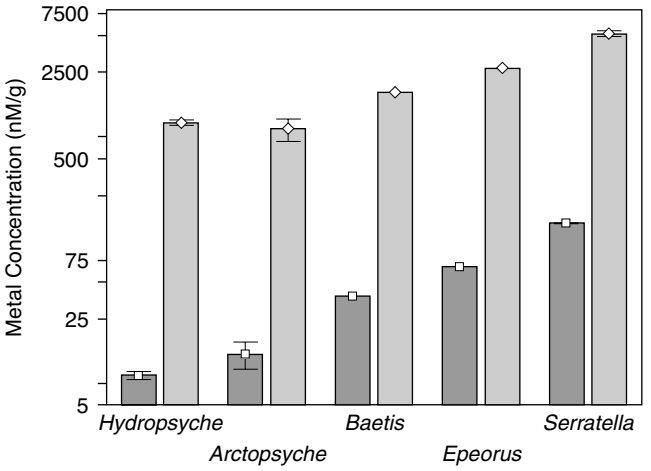

FIGURE 19.10 Difference in copper (triangles) and cadmium (squares) bioaccumulation in the five species followed in Figure 19.9. The species that progressively disappear with increased contamination also bioaccumulate 5 to 10× more metal than the species that appear, by their ecological distributions, to be more tolerant to the metals (the *y*-axis scale is log-converted to allow presentation of both metals).

Observations of ecological change, alone, are inadequate to convincingly demonstrate that metals are causing adverse effects on benthic communities, especially where a great deal is at stake, as at this Superfund site. Other stressors in the watershed (e.g., dewatering, temperature, nutrient inputs) might also correspond with the metal gradient. Mechanistic explanations can improve the explanatory power of a correlation. It is generally accepted that species that bioaccumulate or detoxify metals differently ultimately have different sensitivities to metals (Rainbow, 2004). Cain et al. (2004) showed that bioaccumulation was reduced and detoxification capabilities were enhanced in *Hydropsyche* species and *Arctopsyche grandis* (species deemed tolerant from their distribution in the river) (McGuire, 1999). Bioaccumulation was considerably greater in species typically absent in contaminated waters, including such mayflies as *Serratella tibialis* and *Timpanoga* species (Figure 19.10). Some sensitive species (e.g., *S. tibialis*) do not sequester metals as efficiently into detoxified fractions within their cells (metal-specific binding proteins or intracellular granules) as do the *Hydropsyche* (Cain et al., 2004). Those species (especially mayfly species) that tend to accumulate high concentrations of internal copper and cadmium in forms available for binding to sites active in causing toxicity also tend to be absent from contaminated sites. These findings specifically linked species absences with the likelihood of metal effects.

In contaminated areas, macro-invertebrate drift can increase, community and microbial respiration can decline, and leaf litter breakdown declines (Carlisle and Clements, 2005). Carlisle and Clements (2003) concluded that total production attributable to algae and animal prey declined in contaminated streams. Impairment of moderately sensitive species can include effects as subtle as changes in predator avoidance (Lefcort et al., 2000). None of these effects has been studied in the Clark Fork; nevertheless, such results suggest that fish inhabiting a metal-contaminated stream must depend on a disturbed benthic community for their food—one that is a contaminated food source, less productive, less diverse, and missing attributes of likely (but usually unquantified) importance to fish diversity and productivity.

Effects on Fish

As noted above, the traditional approach to evaluating risk to fish is a comparison of ambient dissolved concentrations against the concentration of metal that elicits effects in laboratory tests. Verification of effects from conditions in the water body are not required for regulatory compliance but are ultimately desirable to justify policy judgments. Interpreting effects on fish in the field is complex, however, and requires a suite of repeated observations building to a systematic body of evidence pointing toward cause and effect. Useful lines of evidence include documented fish kills, *in situ* toxicity testing, population

surveys of wild fish, evidence of exposure in the wild, evidence of toxicity from foods from the water body, and biochemical signs of harm in survivors. It is unlikely that any single experiment or measurement can fully explain the metal effects. Fish kills and effects on young trout held *in situ* were discussed earlier. Other lines of evidence supporting metal effects also have been systematically explored in the Clark Fork.

Abundance and Diversity of Trout Are Reduced

Trout are native to the upper Clark Fork River, as elsewhere in the Rocky Mountains of Montana. A history of depauperate populations provides the first-order suggestion that mine wastes may have affected trout populations in the Clark Fork River. For nearly a century, the Upper Clark Fork contained no trout because of the hazardous materials released by mining, milling, and smelting operations (Johnson and Schmidt, 1988). Even though fish began to reappear in the Clark Fork River in the 1950s, periodic kills were indicative of continued stress. Fish returned after such episodes, probably because of immigration from tributaries, but effects on abundances were likely.

Modern populations of wild trout are less abundant and less diverse than expected, but identifying the cause is challenging. Historical data do not adequately define baseline conditions. A substantial amount of time has passed since the release of hazardous substances began. Possible control areas upstream from impact areas do not exist because the headwaters of Silver Bow Creek and the Clark Fork River have been contaminated with hazardous substances. Finally, separation of mining impacts from the influences of other stressors can be problematic. Despite these complexities, a consistent body of evidence points to metal contamination as a major cause of the disturbed fish populations in the Clark Fork River.

With the advancement of electrofishing gear, the State of Montana began conducting trout population estimates in the Clark Fork River in the late 1960s, using the adjusted Petersen mark–recapture method (Chapman, 1951). The Peterson method makes a number of assumptions, most of which were likely violated in the Clark Fork River (Hillman, 1991). The population estimates, therefore, may overestimate true population sizes (systematic error); however, trends in numbers may be valid if there is continuity in sampling protocols. Estimates from the two most common upstream sites (immediately below the ponds and below Deer Lodge) rarely recaptured small brown trout, so the population numbers are for brown trout larger than 6 inches. Sampling was sporadic spatially and temporally; data are most complete for spring sampling, so those are reported here.

Figure 19.11 shows the pond anomaly in fish abundance, in context. There are no fish upstream of the ponds. High abundances occur in one very short reach of the river, but below the pond anomaly trout abundance is low throughout the Clark Fork. The Deer Lodge site averaged 225 trout per mile from 1967 to 1998. The highest density of brown trout was 356 trout per mile in 1987; 89 trout per mile were found in 1989. During spring 1987, the State of Montana conducted population estimates from the settling ponds to Milltown Dam, at 80 contiguous sections, which they combined into 31 sections to improve density calculations. Trout numbers were less than 500 trout per mile near Deer Lodge and less than 100 trout per mile between Flint Creek and Rock Creek. Trout numbered less than 500 trout per mile downstream below the confluence of Rock Creek.

Trout numbers for the Clark Fork are considerably lower than in its tributaries (Knudson, 1984), although physical conditions differ among the streams. The Blackfoot River has the highest trout populations in the upper Clark Fork system, numbering about 2500 trout per mile. Warm Springs Creek and Rock Creek average about 1500 trout per mile. Flint Creek and the Little Blackfoot River support about 1000 trout per mile. Knudson (1991) compared numbers of trout in the Clark Fork River with other Montana streams, including the Madison River downstream from Ennis which has 4600 trout per mile.

Clark Fork tributaries and other trout streams in Montana also contain a greater diversity of trout species than the upper Clark Fork River; for example, the Blackfoot River, Rock Creek, and the Little Blackfoot River support populations of brown, rainbow, cutthroat, bull, and brook trout (Hillman and Chapman, 1995; Hillman et al., 1995; Knudson, 1984). Willow Creek supports brook and cutthroat trout, while German Gulch Creek supports cutthroat, brook, and brown trout (Camp, Dresser, and McKee, Inc., 1991). Lower river tributaries, such as the Bitterroot, Flathead, and St. Regis rivers, support brown, rainbow, brook, cutthroat, and bull trout. Outside the Clark Fork watershed, the Madison, Gallatin, Yellowstone, and

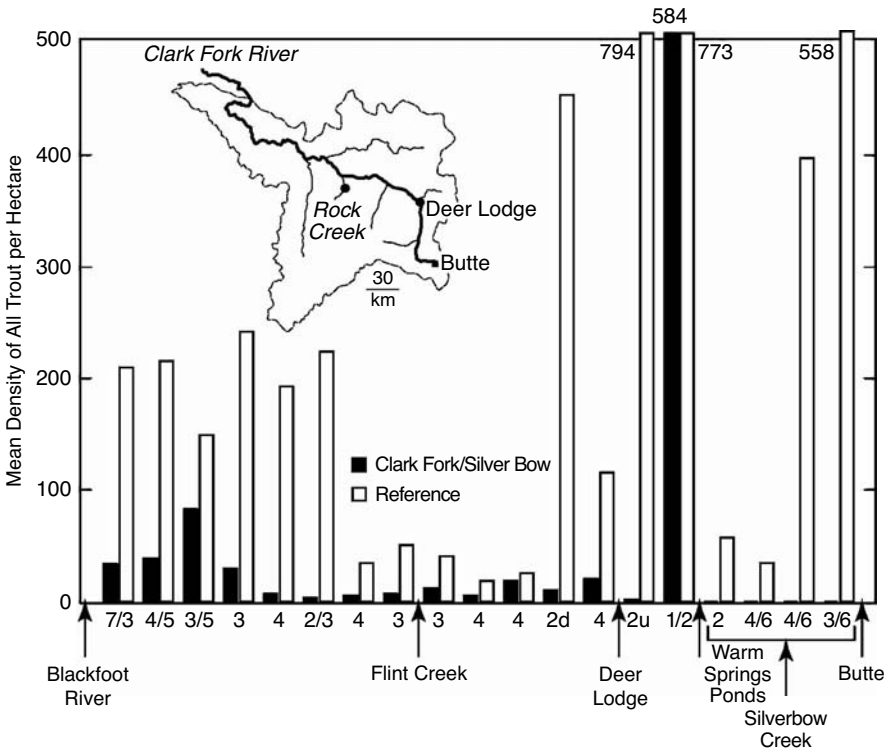

FIGURE 19.11 Mean densities of all species of trout combined in Silverbow Creek and the Clark Fork River, compared to densities in carefully matched reference reaches in other Montana streams. Reference segments were identified in the Big Hole, Ruby, and Beaverhead rivers and in the Rock, Flint, and Bisson creeks in Montana. (Adapted from Hillman, T.W. and Chapman, T.W., in *Aquatics Resources Injury Assessment Report*, *Upper Clark Fork River Basin*, Lipton, J. et al., Eds., Report to the State of Montana Natural Resource Damage Program, Helena, MT, 1995; Hillman, T.W. et al., in *Aquatics Resources Injury Assessment Report*, *Upper Clark Fork River Basin*, Lipton, J. et al., Eds., Report to the State of Montana Natural Resource Damage Program, Helena, MT, 1995.)

Missouri rivers support brown, rainbow, brook, and cutthroat trout. In contrast, Silverbow Creek supports no fish. The Upper Clark Fork River supports almost entirely brown trout, with rainbow trout first appearing in abundance 170 km from the mining and smelting activities, below the confluence of Rock Creek. Typical native species—bull trout (*Salvelinus confluentus*) and westslope cutthroat trout (*Oncorhynchus clarki lewisi*)—have been virtually eliminated from the main-stem upper river (Malouf, 1974).

Comparisons of trout abundance could be misleading if large differences in geology, geomorphology, channel conditions, and habitat exist between streams; this was one source of contention in the early discussions of mining impacts. Hillman et al. (1995) conducted studies that compared trout populations in the Clark Fork and Silverbow Creek with reference areas that were matched on several variables. Silver Bow Creek and the Clark Fork River were first classified in terms of ecoregion, geology, geomorphology, and habitat conditions. This resulted in 19 discrete survey segments, 4 in Silver Bow Creek and 15 in the Clark Fork River. References were then selected based on similar ecoregion, geology, land type association, valley bottom type, stream state type, elevation, valley grade, stream sinuosity, stream grade, dominant substrate, riparian vegetation type, and channel type. Habitat surveys in both Silver Bow and Clark Fork and the reference segments were also included in the comparisons. Habitat measurements included channel width; wetted perimeter width; riffle, run, and pool widths; pool rating; bank angle; average and thalweg depths; substrate; bank cover; vegetation overhang; canopy cover; bank alteration; woody debris; sun arc; and bank undercuts. In addition, basic water-quality characteristics were determined (dissolved oxygen, dissolved organic carbon, temperature, nitrate/nitrogen, conductivity, hardness, and alkalinity). Reference segments were identified within and outside the Clark Fork watershed.

Underwater observations (via snorkeling) and electrofishing were used to estimate trout numbers and biomass in 100-m comparable segments. Removal–depletion electrofishing was used where poor water clarity precluded the use of snorkeling, as well as for validating snorkel estimates. In 1991, reference segments contained significantly more juvenile and adult trout (brown and rainbow trout) and more biomass than Silverbow Creek and the upper Clark Fork (Figure 19.11). Reference trout densities averaged 5.3 times greater than densities in contaminated segments. The Physical Habitat Simulation Model developed by the U.S. Fish and Wildlife Service was used to address differences in habitat (Hillman et al., 1995). This model calculated the weighted useable area (WUA) of trout habitat for a given segment. Numbers and biomass were divided by the WUA to account for differences in habitat. A comparison of adjusted trout populations in both Silver Bow and Clark Fork and reference segments showed the same pattern: Adjusted adult and juvenile trout densities in reference segments were 5.8 times greater than densities in the Silver Bow and Clark Fork segments. Hillman and Chapman (1995) repeated their studies in 1994 with similar results. Total trout densities in the Clark Fork segments ranged from 7 to 188 trout per hectare. Trout populations in reference segments ranged from 39 to 528 trout per hectare. In addition, although trout densities fluctuated over the course of the summer in each of the four Clark Fork segments, similar patterns of change were observed in the reference segments, and the difference between Clark Fork and reference segments remained significant at all times.

These careful comparisons come as close as possible to documenting that population differences are not accounted for by differences in trout habitat. Overall, the population studies substantiate the uniquely low abundance and diversity of trout in the Clark Fork River compared to streams affected by stressors typical of Montana streams but unaffected by mine wastes.

Metal Contamination Is Bioavailable to Trout

Corresponding to the elevated concentrations of metals in water, sediment, and benthic macroinvertebrates, the resident fish in the Clark Fork River also contain elevated tissue metals. Farag et al. (1995) documented elevated arsenic, cadmium, copper, and lead in the gill, liver, kidney, pyloric ceca, stomach, large intestine, stomach contents, and whole fish of brown trout from two sites in the Clark Fork, compared to brown trout collected from two reference sites. Tissue concentrations of copper were greater than 2300 μg/g (dry wt) in livers and greater than 1250 μg/g in the gill, pyloric ceca, and stomach tissues of brown trout sampled near Warm Springs Ponds (Figure 19.12). Similar concentrations were measured in April of 1989 (Phillips and Spoon, 1990) and in August and November of 1991. In earlier years (Dent, 1974), the concentrations were greater than the concentrations reported above. The elevated metal concentrations in specific tissues of fish from the Clark Fork River indicated that resident fish in this river system were exposed to bioavailable copper and acquired a tissue dose of metals in specific organs. Farag et al. (1995, 1999) suggested that a liver concentration between 238 and 480 μg Cu per g (dry wt) was also detrimental to growth and reproduction (Table 19.2).

Effects of Metals Via the Diet Are More Severe Than Effects from Chronic Dissolved Exposures

Traditional toxicity tests do not account for the possibility that fish are exposed to metal contamination via dietary intake. As noted previously, invertebrates in the Clark Fork River have 2 to 100 times greater cadmium, copper, and lead levels than those collected from tributaries and are an important source of food for trout in the Clark Fork. Woodward et al. (1994) fed early-life-stage trout invertebrates collected from near the Clark Fork headwaters and from 85 km downstream. After eating the contaminated diet, the fish showed elevated concentrations of products of lipid peroxidation and histological abnormalities (effects on hepatocytes, pancreatic tissue, and the mucosal epithelium of the intestine), as well as reduced growth and survival (Farag et al., 1994; Woodward et al., 1994, 1995).

This experimental approach was reproduced in a series of experiments using different trout species and with benthos from different contaminated rivers. Similar toxicological responses usually were observed: reduced survival (when diets were collected from the Coeur d'Alene River in Idaho), decreased growth, reduced feeding activity, and histopathological abnormalities (Farag et al., 1999; Woodward et al., 1994, 1995). All of the responses were associated with the bioaccumulation of metals in the tissues of the fish.

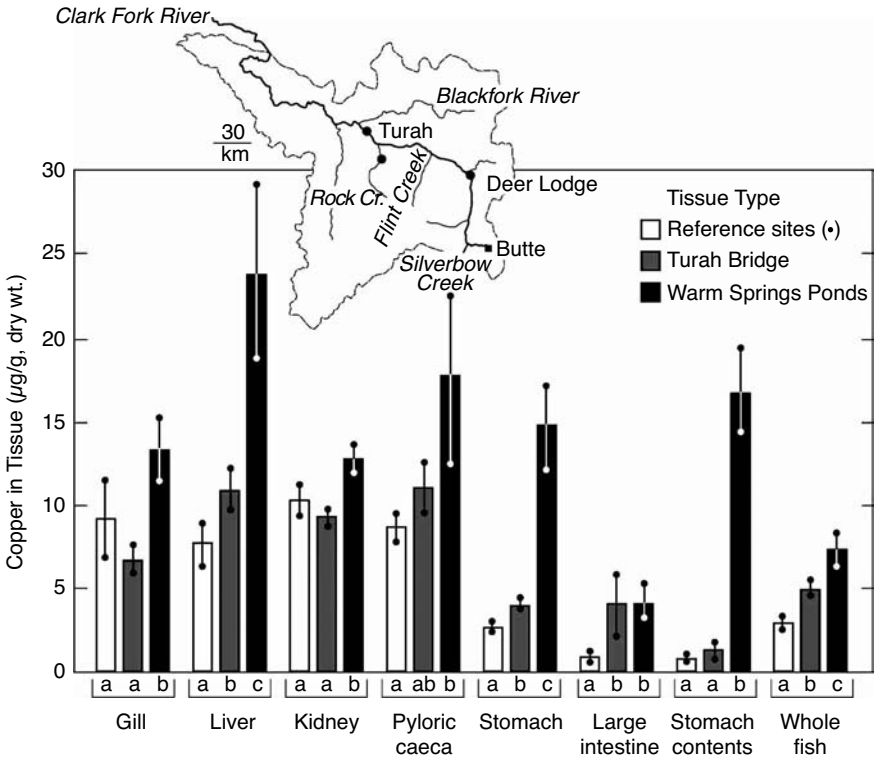

FIGURE 19.12 Bioaccumulation of copper in different tissues of brown trout (*Salmo trutta*) from below Warm Springs ponds and from Turah, on the Clark Fork River, compared to fish from uncontaminated reference sites. (Adapted from Farag, A.M. et al., *Can. J. Fish. Aquat. Sci.*, 52, 2038–2050, 1995.)

The lipid peroxidation observed experimentally was also observed in the liver, pyloric ceca, and large intestine of brown trout resident in the Clark Fork (Farag et al., 1999). Metals that exist in more than one valence state (e.g., copper) can initiate lipid peroxidation (Wills, 1985) by interacting with sulfhydryl groups and oxygen or by inhibiting important antioxidant enzymes. This may ultimately result in tissue damage or cell death (Sokol et al., 1990), when fatty-acid side chains in cell membranes are the targets (Halliwell and Gutteridge, 1985). Brown trout from the upper Clark Fork River also contained copper inclusions in hepatocytes and vacuolation of hepatocyte nuclei. When energy is diverted from cell metabolism to detoxify metals, cell disruption is noted by both vacuolation of nuclei and the elevated lipid peroxidation.

The experiments reported above were somewhat controversial because nutritional content and species composition of the diet were not controlled. Mount et al. (1994) repeated the dietary exposure experiments with rainbow trout (*Oncorhynchus mykiss*), but they used brine shrimp (*Artemia*) as a food source. After 60 days, they could attribute no effects on growth or survival to the diet. Woodward et al. (1995) and Mount et al. (1994) both, however, documented effects on the intestinal tract of fish fed diets contaminated with metals. Gut impaction was observed in 3 to 9% brown trout, and constipation was observed in nearly 50% of the trout fed diets from the uppermost Clark Fork River. Mount et al. (1994) found that 7 of 18 mortalities observed were associated with actual rupture of the body cavity. Hansen et al. (2004) controlled the nutritional content of the diet by feeding rainbow trout worms (*Lumbricus variegatus*) grown in Clark Fork sediments. They found histopathologic abnormalities in treated animals, along with unambiguous inhibition of growth. The growth reduction was associated with reductions in conversion of food energy to biomass (rather than reduced food intake) and correlated with arsenic bioaccumulation.

Metals may also exert effects on fish by changing the types of prey species that are present and thus changing the dietary sufficiency available to fish in the system. A number of metal-sensitive species are missing from the benthic community of the Clark Fork in the uppermost river. In the dietary exposure experiments compared above, the concentrations of essential amino acids were greater in *Artemia* than in the collection of invertebrates collected from there (Farag et al., 2000). Where diet caused some of the differences between the toxicity experiments, it may reflect deficient nutritional conditions in the river.

The indirect effect of metals on the diet of fish in a stream could be a fruitful avenue of investigation in the future, but uncertainties about such explanations have added to the controversy regarding diet as a source of toxicity. Dietary toxicity is a complex subject and one of some controversy in the area of metal ecotoxicology, even though the importance of dietary exposure is unambiguous (Luoma and Rainbow, 2005). It is difficult to discount the experiments that most closely simulate the diet of trout in the Clark Fork, but a USEPA risk assessment (1999) concluded that the effects of dietary exposure in the Clark Fork River were ambiguous. The Agency's lack of experience with this exposure route was probably a contributing factor in that decision. Despite the controversy, it is clear that assessment of metal exposure to fish populations in natural systems must include evaluation of dietary as well as waterborne metal contamination (Farag et al., 1999).

Pulse Inputs of Metals Can Also Affect Fish Abundance and Diversity

Marr et al. (1995a,b) performed laboratory experiments to determine the effects of pulse and chronic exposure of metals in the water column on fish. The pulse concentrations simulated the mixture of metals in the Clark Fork River during fish kill events (Phillips, 1995). Metal concentrations were increased over a 1- or 2-hour period, held constant for 6 or 4 hours, then decreased over a 1- or 2-hour period. The post-pulse mortality was monitored, and an LC_{50} for each experiment was calculated. Alkalinity, pH, and conductivity were kept constant, increased, or decreased during the pulse. Marr et al. (1995a,b) found that fry and juvenile trout were equally sensitive to the pulse exposures; fish that died during these experiments experienced losses of potassium and calcium (≥27 and ≥33%, respectively) but not sodium. Rainbow trout fry were more sensitive than brown trout fry when elevated metals were present with depressed hardness and pH, conditions that mimic rainstorm events. The authors concluded that thunderstorm events in the Clark Fork River that supplied metals-rich acidic runoff to the river would especially limit the survival of rainbow trout. Brown trout fry, however, were more sensitive than rainbow trout fry to 8-hour exposures with constant hardness and pH, so simple chronic exposure alone did not explain the dominance of brown trout in the upper river.

Acclimation Differences Explain the Reduced Diversity of the Trout Community

Acclimation of resident fish to elevated concentrations of metals may cause an increased tolerance to metals and aid survival of some species. Marr et al. (1995a,b) tested tolerance and resistance in naïve and metals-acclimated brown and rainbow trout. Tolerance levels were determined from 96-hour median lethal concentration (LC_{50}) or incipient lethal level (ILL). Resistance was measured as the median lethal time (LT_{50}) and mean time to death (TTD). Naïve hatchery rainbow trout were more tolerant to acute metal exposures (greater LC_{50}) than naïve brown trout, but brown trout that were acclimated to sublethal concentrations of cadmium, copper, lead, and zinc were most resistant and survived the metal exposures for the longest periods of time. Marr et al. (1995a,b) also documented an increase in metallothionein (MT) in brown trout, but not rainbow trout, following acclimation. Resident brown trout from the Clark Fork River also induced this metal-binding detoxification protein (Farag et al., 1995); thus, a better ability to acclimate via induced detoxification of metals may also explain why brown trout survive better than rainbow trout in the Clark Fork. Although MT induction may aid brown trout survival, it was associated with a reduction in growth during the acclimation. Others have documented reduced growth as a physiological cost associated with metal detoxification by metallothionein induction (Dixon and Sprague 1981a,b; Marr et al., 1995a,b; Roch and McCarter, 1984). Such costs for survival may explain why brown trout resident in the Clark Fork River have a smaller length at annulus compared to reference populations (Tohtz, 1992).

Fish Avoid Natural Waters That Are Metal Contaminated, Affecting Availability of Habitat

Behavioral avoidance of unfavorable concentrations of metals such as copper and zinc may be an additional cause of reduced fish populations in natural systems. Behavioral avoidance is particularly important because it can occur at concentrations lower than those causing effects on survival and growth. It may limit fish populations by displacing them from preferred habitats. Copper in the Clark Fork River is at and above the concentrations that cause avoidance by trout. In experiments, brown trout and rainbow trout avoided conditions simulating the presence of copper and other metals in the river (Hansen et al., 1999; Woodward et al., 1995). Rainbow trout were more sensitive than brown trout. Even after 45 days of acclimation to a metals mixture that simulated the Clark Fork River, rainbow trout preferred clean water over water with elevated concentrations of metals. This finding may also favor the presence of brown trout over rainbow trout in the upper river.

Although detection and avoidance of undesirable substances have been documented in the laboratory, wild populations of fish are influenced by numerous other factors. Goldstein et al. (1999) used radiotelemetry and a migrating population of Chinook salmon to determine the response of a wild population of fish to the presence of metals. Salmon were transferred from their natal stream to a point 2 miles below the confluence of the North Fork and the South Fork of the Coeur d'Alene River. The South Fork has elevated concentrations of metals (mainly cadmium, lead, and zinc with some copper) from historical mining activities, and the North Fork is relatively pristine. The fish were tagged with radiotransmitters and allowed to move upstream. Although other influential variables were operative—flow, temperature, general water quality, and cover—the higher metals concentration in the South Fork tributary appeared to be the factor of greatest difference. The majority (70%) of the fish tagged selected the North Fork. The results of this experiment, coupled with a companion laboratory study showing that fish avoid the metal mixture in the Coeur d'Alene River, support avoidance as a factor in reduced fish abundances.

Conclusions

In this chapter, we show that large-scale dispersion of sediment and metal contamination is a consequence of base-metal mining operations that do not carefully manage their wastes. The ultimate effects of such mining activities on fish populations are determined by the types of wastes, how those wastes are contained, and the hydrologic and ecological characteristics of the affected river system. In the Clark Fork, circumneutral contaminated sediments are dispersed for hundreds of kilometers, contributing contamination to the water column and the food web.

It is a substantial challenge to document metal dose in nature and to show unambiguously that metal dose is related to biological effects. Simple, linear dose–response curves are not common in field studies of fish effects because of the multiple metal exposure routes, complicated spatial exposure regimes on scales smaller than hundreds of kilometers, temporally variable exposures, and confounding stressors. Traditional measures of metal effects have yielded ambiguous results in the Clark Fork River; for example, toxicity tests that rely on surrogate species and water exposures do not suggest toxicity in the river. Pulses of metal input seem quite feasible but are difficult to document in conjunction with toxicity (other than in historic fish kills). Nevertheless, the body of evidence, integrated with observations from the river itself, consistently shows that mine wastes are a primary influence on fish in Clark Fork.

An intuitively obvious agreement exists between fish distributions and metal contamination in all compartments of the environment. Lower fish diversity and fewer numbers of fish, compared to carefully documented reference areas, accompany the occurrence of mining-related metal and sediment contamination. Other characteristics of the fish community are also consistent with metal exposures and what is known about metal effects. Above Rock Creek, brown trout dominate the community. Laboratory studies show that brown trout acclimate more effectively to metal contamination and are better able to detoxify metals via metallothionein induction than are rainbow trout. They also are less sensitive in their avoidance response.

The dietary pathway of metal exposure (e.g., via contaminated invertebrates) appears to be especially important for Clark Fork fish. Reduced contamination of sediments and invertebrate prey indicates that

the fish diet is not very contaminated immediately below upstream waste containment ponds. This unexpected feature in the distribution of contamination is accompanied by the greatest numbers of fish in the system. A rapid increase in invertebrate contamination downstream 5 to 10 km from the Warm Springs Ponds is attributable to inputs from contaminated banks and the floodplain (probably with contributions from the hyporheic zone). This is accompanied by a rapid decline in fish abundance. Invertebrate communities are also disturbed in this region. Metal-sensitive species are missing through much of the Upper Clark Fork, and those species missing are the strongest bioaccumulators of copper or cadmium or are the animals with the least capabilities for detoxification. Suborganismal indications of stress similar to those generated by dietary exposure of trout to metal contamination are found in surviving fish populations in the Clark Fork. Surviving fish appear to be stressed in some of the same ways that metal-contaminated diets stress fish in the laboratory, further supporting the importance of metals as a stressor in the system.

Important mechanistic details, however, have not been fully resolved. What is the most important risk to fish: acute toxicity episodes, chronic stress and mortality to adults (e.g., from a contaminated diet), toxicity to juveniles, failure of early life stages to survive, effects on reproduction? More needs to be understood about why some fish species are absent and others are not. Specifically, the vulnerability of native cutthroat and bull trout should be studied. The intriguing possibility that the simplified benthic community that accompanies metal contamination is nutritionally inadequate for some fish species remains to be fully studied. In short, mine wastes manifest their effects in both intuitively obvious and very complicated ways in a contaminated river. Contentious debate is reduced as knowledge grows, but full consensus on metal effects awaits better mechanistic understanding. There is value in integrating long-term, multidiscipline, persistent field and laboratory investigations to unravel the mechanisms behind the complex responses that mine wastes create.

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20

Toxicology of Synthetic Pyrethroid Insecticides in Fish: A Case Study

Joel R. Coats

CONTENTS

Introduction

The development of photostable synthetic pyrethroids as broad-spectrum commercial insecticides around 1980 represented a new threat to fishes. Naturally occurring pyrethrins, from a Eurasian species of chrysanthemum, had been used for decades for control of insects in homes, restaurants, barns, and gardens and on pets, livestock, and stored grain. They were selective, safe, and nonpersistent (their half-lives in sunlight are measured in minutes or hours). They posed very few nontarget effects or residue problems. Although they are acutely toxic to fish, very few accidental poisonings occurred because they are not registered for aquatic uses, and they seldom have enough persistence to reach water from a normal application.

After decades of developmental efforts, several chemical companies were successful in synthesizing, patenting, and formulating several important photostable pyrethroid insecticides for commercialization. Through stepwise chemical substitution of three or four labile groups in the basic pyrethrin molecule, the agrichemical company chemists invented numerous more stable and more potent synthetic analogs

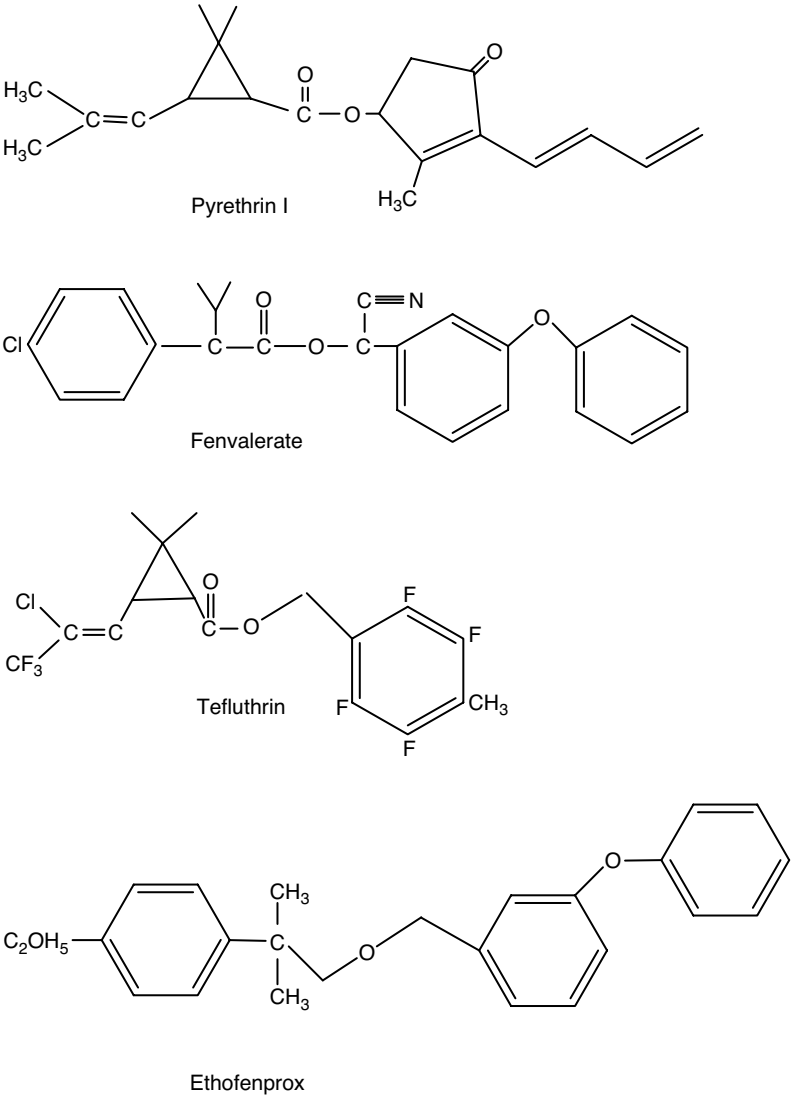

FIGURE 20.1 Structures of one natural and three synthetic pyrethroid insecticides.

of the natural pyrethrins. The use of more stable side-chains and rings, as well as the inclusion of chlorine, bromine, and fluorine, resulted in synthetic pyrethroids that were much less susceptible to oxidation, hydrolysis, and photolytic reactions. The new types of pyrethroids were labeled *photostable*, but they persisted for much longer times in water and soil, as well as on plant surfaces. Figure 20.1 shows one natural pyrethrin and three synthetic analogs that all generate essentially the same toxic symptoms and effects, despite quite divergent structures.

When the chlorinated hydrocarbon insecticides were introduced 60 years ago, accidental fish kills became commonplace. DDT, dieldrin, endrin, heptachlor, chlordane, toxaphene, endosulfan, and other related toxic, lipophilic, and persistent pesticides are extremely toxic to fish, with LC_{50} values in the range of micrograms per liter (parts per billion) to nanograms per liter (parts per trillion). There were also serious effects from chronic exposures to the ubiquitous low-level residues in water and sediment. The exceptional persistence and lipophilicity of the chlorinated hydrocarbons led to numerous deleterious effects on reproduction, growth, and survival. Biomagnification of the residues occurred upward through trophic relationships; simple uptake across gills also increased concentrations in fish tissues.

Environmental concerns eventually resulted in banning or restricting most of the persistent bioaccumulating chlorinated hydrocarbons.

The replacement pesticides were primarily organophosphates and carbamates, which are very degradable and are especially susceptible to pH-dependent hydrolysis in aquatic systems. Water solubilities of these compounds are generally 3 to 6 orders of magnitude higher, and LC_{50} values are more commonly in the milligram-per-kilogram range. Although occasional fish kills still occur, the acute and chronic impacts on fish populations have been greatly diminished since these degradable cholinesterase inhibitors have replaced the chlorinated hydrocarbons. In the last 20 years, the organophosphates and carbamates have been scrutinized more and more closely due to acute and chronic toxicity problems in birds and mammals, including humans. The Food Quality Protection Act is hastening their phase out; for example, chlorpyrifos and diazinon have been banned for certain uses in homes, gardens, and lawns.

The emergence of synthetic pyrethroids as the primary replacements for the acutely toxic organophosphates and carbamates brought valid new concerns about fish toxicity. Pyrethrin-type molecules have an innate toxicity to fish, and the synthetic pyrethroids represent analogs that have much longer persistence and extreme lipophilicity. Initial toxicity testing revealed LC_{50} values that were as low as those for many chlorinated hydrocarbons against numerous species and stages of fish. The symptoms of toxicity are distinctive, and death is violent. Questions of acute and chronic effects, as well as potential bioaccumulation, raised concern as the pyrethroids became the predominant class of insecticide employed in all of agriculture. Researchers have now addressed many of the questions regarding toxicity, uptake, and tissue residues; this chapter presents the current state of our knowledge of the toxicology of synthetic pyrethroids in fishes, and it provides a putative answer to the question of why pyrethroids are so much more toxic to fish than to birds and mammals.

Toxicity

Synthetic pyrethroids are generally extremely toxic to fish in the standard laboratory 96-hour LC_{50} tests or in 48-hour bioassays, both of which are considered acute toxicity bioassays. As might be expected, numerous factors can modulate the toxicity, including formulation, water parameters, time, temperature, and properties of the chemical being tested. Many synthetic pyrethroids have 96-hour LC_{50} values of less than 1 µg/L, while chronic toxicity can be recorded at one to two orders of magnitude lower than that (Bradbury and Coats, 1989b). It is important to note that fish toxicity studies vary widely in their methodology (e.g., static conditions vs. flow-through exposures; nominal concentrations added to the water vs. measured concentrations). The quality of the test conditions, including exposure, governs to a great extent the validity of the results.

Toxic Mode of Action

The principal mode of action for pyrethroid insecticides in fish is assumed to be the same as the toxic action in insects (Holan et al., 1984) and in mammals. In nerve axons, the sodium gates open briefly to facilitate depolarization of the membrane, which constitutes an action potential that is propagated down the neuron. The pyrethroid insecticide binds to a receptor at the sodium gate of the neuron and prevents it from closing fully (Figure 20.2). The resulting steady leakage of sodium ions into the neuron creates a less stable resting state, and the neuron is susceptible to repetitive firing of the nerve, which leads to hyperactivity, tremors, and tetany (Motomura and Narahashi, 2000; Narahashi et al., 1998). DDT also acts at the sodium gate, but at a different site. Similarities and differences in modes of action and quantitative structure–activity relationships have been addressed (Coats, 1990). Alternative or secondary modes of action have also been put forth for pyrethroids. Calcium channels have been shown to be affected adversely (Clark, 1986; Clark and Brooks, 1989), and the inhibition of calcium and calcium/magnesium ATPase enzymes has also been considered to be a potential toxic effect of pyrethroids (Clark and Matsumura, 1982). GABA-gated chloride channels have also been studied (Bloomquist and Soderlund, 1985; Seifert and Casida, 1985) for the malfunctions that result from exposure to pyrethroid insecticides. The two classes of pyrethroids, type I and type II, are distinguished by their differences in symptomology.

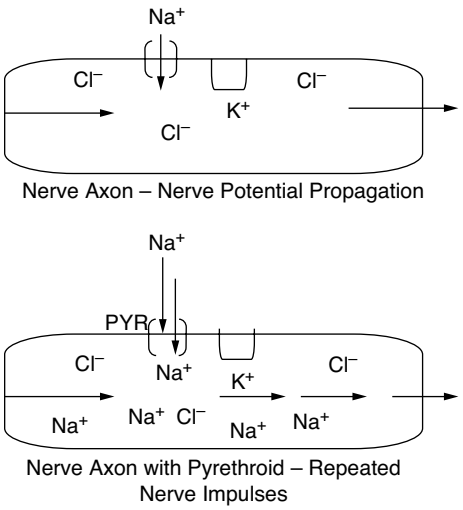

FIGURE 20.2 The mechanism of neurotoxic action of a pyrethroid at the sodium channel of the nerve axon prevents complete closing of the sodium gate, which allows constant leakage of Na^+ ions, which leads to hyperexcitability in the neuron.

Mammalian studies on the toxicodynamics of pyrethroids have shown that the type I pyrethroids cause primarily hyperactivity and tremors, while type II pyrethroids cause writhing, seizures, and choreoathetosis. Both types primarily affect the sodium channels and effect hyperpolarization of the axons, but the type II compounds produce longer sodium currents and cause blocking of nerve conductance. In cockroaches, symptomological differences also exist between the two types of pyrethroids. Research on birds and fish has not generally revealed distinct differences in toxic symptoms, but it is possible that they also respond differently, at some level, to the two classes of pyrethroids. The structural differences between type I and type II pyrethroids are simple; type II pyrethroids have an α-cyano group at the benzylic carbon of the alcohol portion of the ester. In Figure 20.1, pyrethrin I, tefluthrin, and ethofenprox are type I pyrethroids, and fenvalerate (and its commercially important resolved *S*,*S*-isomer) represents the type II class. Soderlund et al. (2002) have provided a review of the toxic modes of action of pyrethroid insecticides.

Toxic symptoms in fish (fathead minnows) have been observed in acute toxicity tests with fenvalerate (Bradbury et al., 1985). Swimming near the surface, darting, hyperactivity, and bursts of rapid swimming eventually led to violent whole-body contractions. The toxic syndrome was investigated in considerable physiological detail in adult rainbow trout (Bradbury et al., 1987a). Increases in cough rate and mucous secretion by the gills were the first signs of toxicity noted. Fine tremors developed, with the tremors slowly becoming more severe and eventually progressing to seizures with twisting and thrashing. Histopathology of the gills after death revealed numerous aneurisms and necrotic cells. Major detrimental impacts on heart rate, ventilation rate, and blood chemistry have also been described in the acute toxicosis phase for the trout.

Chronic toxicity and sublethal toxic effects have been demonstrated at concentrations much lower than those that elicit acute toxic effects. Chronic toxicity endpoints such as growth and reproduction have revealed very low no-observable-adverse-effect levels (NOAELs) for several pyrethroids, with the early life stages showing great susceptibility to impaired growth rates. Behavioral toxicity has been reported in the form of swimming abnormalities (hyperactivity, loss of equilibrium, abnormal lateral flexure) (Glickman et al., 1982; Rice et al., 1997).

Factors Influencing Toxicity

The acute toxicity of pyrethroids to fish was traditionally measured in standardized water or in filtered or purified water. Many studies have demonstrated the extraordinary toxicity of photostable synthetic pyrethroids to fish; in many cases, they are as toxic as organochlorine insecticides such as DDT, dieldrin,

endrin, and chlordane. Initial field trials, however, showed the pyrethroids to be less potent than expected from lab studies. It was determined that the pyrethroids, with their extremely low water solubility and high affinity for particulate matter in solution, did not remain bioavailable for uptake by the fish in the field ponds. When the pyrethroid molecules bound to the suspended solids or the sediment, the resultant toxicity was orders of magnitude less than predicted by the clean-water assays. A similar effect was reported for a dose–response experiment in mosquito larvae (Coats et al., 1989). Some studies have investigated interactions between insecticides and particulate matter in the water and their effect on toxicity (Coats, 1980; Coats et al., 1989; Herbrandson et al., 2003). Mixtures of chemicals in the aquatic systems can also impact organisms in ways that are difficult to predict (Lydy et al., 2004); pyrethroids have been demonstrated to contribute to greater than additive toxicity in fish (Denton et al., 2003).

Formulation

One of the early indicators of the significance of the bioavailability of pyrethroids in fish toxicity tests was revealed in acute toxicity tests that compared technical-grade synthetic pyrethroids with their emulsifiable concentrate (EC) formulations (Coats and O'Donnell-Jeffery, 1979). The 24-hour LC_{50} values for rainbow trout in static-exposure bioassays were noted to be two- to ninefold lower for the formulated materials compared to the pure technical-grade active ingredients. The emulsifiable formulation kept the pyrethroids in solution longer compared to the technical materials, and the pyrethroids quickly adsorbed to the glass (and probably to the outside of the fish), removing them from solution. The tendency for pyrethroids to bind to glass and plastic was later confirmed quantitatively (Sharom and Solomon, 1981). The utilization of a flow-through apparatus provided better comparison of formulated and technical fenvalerate in a toxicity test. The 24-hour LC_{50} values were twofold higher for the EC formulation, but by day 7 the incipient LC_{50} values were demonstrated to be the same. Residues were measured over the time course, and the technical-grade fenvalerate was taken up faster than the EC. The resulting times to mortality were also significantly different such that, for comparable exposure concentrations, the time to death was shorter for the fish exposed to the technical grade (Bradbury et al., 1985).

Water Parameters

The ionic characteristics of the water can exert influence on the toxicity of pyrethroids to fish. Because of the role of ATPases in fish osmoregulation (Dange, 1986) and because pyrethroids had been demonstrated to inhibit ATPases in squid axon (Clark and Matsumura, 1982), it was hypothesized that interference with osmoregulation was a secondary mode of toxic action of pyrethroids in fish. Water hardness was shown to be a factor in bluegill susceptibility to fenvalerate (Dyer et al., 1989). The 48-hour LC_{50} values ranged from 0.9 to 1.9 μg/L for bluegill fry. The LC_{50} values were twofold higher in very soft water (6 mg $CaCO_3$ per L), compared to those obtained from harder water (>36 mg/L). Residue analysis of the living and dead fry showed that neither net uptake rate nor final body burden changed significantly with hardness. When salinity was examined as a possible factor, a 50% increase in toxicity was recorded when salinity was raised from 12.5% to >25% in the bluegill fry (Dyer et al., 1989). A second approach to the question was developed through the utilization of radioactive ions with bluegill and fathead minnow. The uptake and depuration of ^{22}Na, ^{36}Cl, and ^{45}Ca were investigated in individual experiments over a range of fenvalerate concentrations (Symonik et al., 1989). The pyrethroid exposure did result in ionic imbalances for each of the ions, raising the possibility that the osmoregulation system may be stressed by the insecticide, but the whole-body ion analysis method did not allow any more specific conclusions about this effect. Together, the two studies indicated that stressing of the ionic regulation system may be a contributing secondary mode of action of pyrethroids in fish.

Temperature

A notable negative temperature coefficient exists for the susceptibility of fish to pyrethroids. The phenomenon is described as an enhanced toxicity at lower temperatures and had previously been long observed in insect toxicology. It is very unusual for any chemical class to be more toxic at lower temperatures; DDT and the pyrethroids are the major examples known to date.

Suspended Solids and Sediment

Although the toxicity of synthetic pyrethroids in clean water is extraordinary, the observations in field trials have found that pyrethroids have not had as draconian an impact on fish populations as once feared. Their toxicity is dramatically influenced by the presence of particulate matter in the water column, probably through adsorption of the very lipophilic toxicant molecules to the suspended matter, sediment, and possibly dissolved organic matter, as well (Coats et al., 1989; Smith and Stratton, 1986). Bioavailability of the pyrethroid fenvalerate in water was shown to be drastically reduced in bioassays that measured LC_{50} values for mosquito larvae in clean water and four different concentrations of humic acid in the water. Up to a sixfold difference was observed in the 24-hour LC_{50} values, depending on the concentration of humic acid added (Coats et al., 1989).

Toxicokinetics

Considerable research on the toxicokinetics of synthetic pyrethroids has been conducted in fish, in hopes of finding explanations for the extreme susceptibility of fish to this class of insecticides compared to birds and mammals and compared to other biodegradable insecticides (e.g., organophosphates and carbamates):

- Are pyrethroids taken up more rapidly than other organic chemicals by the fish gills?
- Is there an especially effective distribution of pyrethroid residues to the nervous system in fish?
- Are fish deficient in their capability for detoxification of pyrethroids?
- Do fish excretory systems have poor efficiency of elimination of pyrethroids?

Uptake

The most definitive studies of uptake rates by fish gill have been conducted by Jim McKim at the U.S. Environmental Protection Agency (USEPA) lab in Duluth, MN. Working with him, Bradbury and Coats (1989a) studied the uptake efficiency of ^{3}H-fenvalerate in rainbow trout in a passive diffusion model for xenobiotic uptake and distribution (McKim and Heath, 1983). The average uptake efficiency was 28.6%, which was much lower than the majority of other organic chemicals studied in that system. The calculated logP of 7.2 for fenvalerate was similar to that calculated for mirex (logP of 7.5); the gill uptake efficiency for the rainbow trout was also low for mirex (20%). The implication is that mirex and fenvalerate are so lipophilic that they are not rapidly absorbed across the fish gill (Bradbury et al., 1986), and they are both taken up more slowly than the numerous other classes of chemicals tested (McKim et al., 1985) which were less lipophilic. It is possible that an optimum lipophilicity exists for rapid uptake by fish rather than the oft-assumed positive correlation between lipophilicity and uptake rates in fish.

Distribution

The radiolabeled fenvalerate also allowed for study of the distribution of residues within the rainbow trout body. The largest proportion of residues were found in the bile, with the fat deposits next, followed by the liver, gill, kidney, and red blood cells. All of those locations are to be expected for a lipophilic xenobiotic, and the concentration in the brain (21 parts per trillion) was lower than most other tissue levels. The distribution does not seem to especially favor selective partitioning or delivery to the brain (Bradbury et al., 1986).

Detoxification

The biotransformation of several synthetic pyrethroids has been examined; those studied have included fenvalerate, permethrin, and cypermethrin. Identification of the most prevalent metabolites revealed that oxidation products were common, primarily due to ring hydroxylation and side-chain oxidation reactions

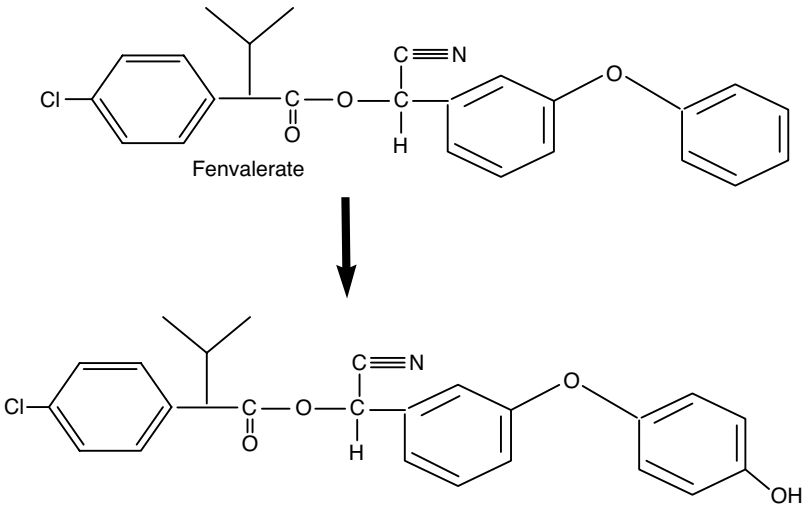

FIGURE 20.3 The primary detoxification reaction for fenvalerate in fish is ring-hydroxylation at the 4′ position which is followed by conjugation to produce a glucuronide conjugate.

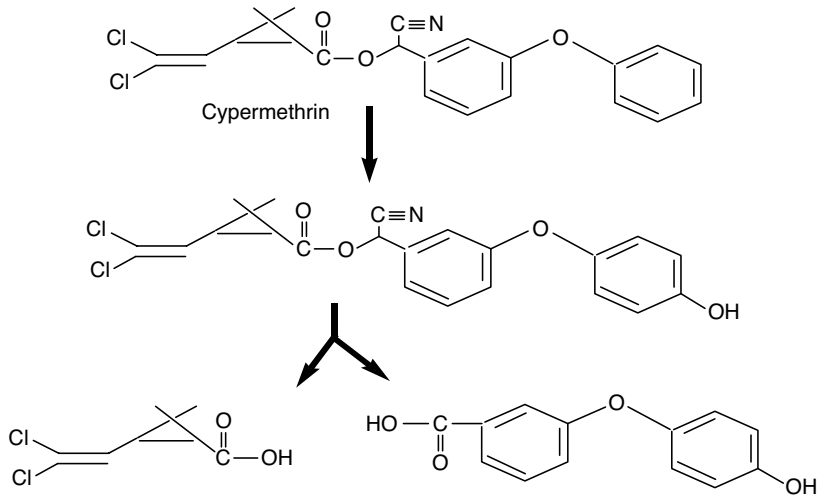

FIGURE 20.4 The metabolic pathway for detoxification of cypermethrin proceeds via the 4′-hydroxylation reaction in fish and higher vertebrates. The hydrolysis reaction shown occurs rapidly in birds and mammals, but not in fish; this deficiency contributes to the enhanced toxicity of pyrethroids in fish.

(Figure 20.3). Products of ester hydrolysis were rarely found, except as reported in one abstract (Figure 20.4). One study on the toxicity of the oxidation products showed that the largely intact ones retained some toxicity, although less than the parent. The alcohol and acid moieties that result from ester hydrolysis are of minimal toxicity to any animals. The importance of esterases for detoxification of pyrethroids by mammals and birds is notable because the metabolite profile in those organisms reveals ample hydrolysis and oxidation products. Studies by Glickman et al. (1982) showed that the exposure of rainbow trout to permethrin did not result in more toxicity when an esterase inhibitor was added, which reflects a lack of esterase involvement in detoxification of the pyrethroid in that species. The fish species investigated by several labs all seemed to be deficient in hydrolysis capability (Bradbury and Coats, 1989a). This is most likely a significant contributing factor for the susceptibility of fish to synthetic pyrethroids.

Elimination

The experiments conducted on the toxicokinetics of fenvalerate indicated that the elimination rate in rainbow trout was much slower than in birds or mammals. After the uptake across the gill membrane, 80 to 90% of the dose taken up was still in the body at time periods of 8 hours to 48 hours after the exposure was stopped. In contrast, research on quail (Bradbury, 1982; Mumtaz and Menzer, 1986) and rats (Ohkawa et al., 1979) found 90 to 100% elimination occurred within 48 hours of administration of a dose. The 10 to 20% of the fenvalerate found in the trout bile between 8 and 48 hours was all present as the glucuronide conjugate of 4′-hydroxy fenvalerate. Any deficiency that fish show in detoxifying or eliminating pyrethroid insecticides could lead to higher concentrations of the parent molecule in their brain. Distribution within the fish body results in relatively small percentages of the parent compound reaching the central nervous system, so any factor that contributes to slower detoxification or excretion of these potent neurotoxicants can enhance the potency in the nervous system by facilitating a small increment in concentration at the site of toxic action. One other question that has to be considered is that of potentially high bioconcentration factors (BCFs) of synthetic pyrethroids in fish. Although the elimination rate is relatively slow, the uptake rate has also been shown to be slow, and sufficient oxidative biotransformation has been observed to preclude grouping these lipophilic, relatively stable synthetic pyrethroids with the older chlorinated hydrocarbons that accumulated to extremely high levels, especially through food chains. Several studies have reported BCFs of 400 to 4000 for fish, which are several orders of magnitude lower than those reported for DDT, dieldrin, chlordane, heptachlor, and endrin. A discussion of the results of several studies is presented in Bradbury and Coats (1989a). The bioconcentration factors for the pyrethroids are not notably higher than those for other, much less toxic, insecticides, including organophosphates and carbamates; the pyrethroid bioconcentration factors probably do not contribute greatly to their extreme potency to fish.

In summary, the toxicokinetics of photostable synthetic pyrethroid insecticides in fishes is substantially different from their toxicokinetics fate in mammals and birds. Some of the differences, especially the lack of hydrolytic detoxification capability and the slow elimination, are consistent with greater toxicity of the pyrethroids in fish.

Toxicodynamics

The effects of synthetic pyrethroids on the nervous system have been studied for decades, but the comparative toxicodynamics have primarily focused on the differences between type I and type II pyrethroids in the mammalian central nervous system and the function and number of sodium gates in the nervous systems of resistant vs. susceptible insects. Additionally, researchers have been curious about the negative temperature coefficient of insects, although it has been documented in other species as well. It is important that a comparative study of fish susceptibility relative to avian and mammalian susceptibility include some consideration of possible differences in toxicokinetics that could contribute to differential susceptibilities. Other questions that may be significant include the following:

- Is the stereoselective toxicity of pyrethroid isomers different for fish than for birds and mammals?
- Do secondary toxic mechanisms of action contribute to the ultra-susceptibility of fish to pyrethroids?
- Are fish nervous systems more susceptible to neurotoxicological effects of pyrethroids compared to avian and mammalian nervous systems?

Stereoselective Toxicity

Insect toxicity is dramatically different for individual diastereomers of pyrethroid insecticides, with some isomers demonstrating incredible potency and their mirror-image isomers showing virtually no toxicity. A similar pattern has been recorded for mammalian LD_{50} values, but little information was available for fish. Some of the limited data in fish depend on the stability of the individual isomers in the water and

TABLE 20.1

Acute Lethality of Fenvalerate and Its Constituent Isomers to Bluegill

Isomer	48-hr i.p. LD_{50} (mg/kg) (95% Confidence Interval)	Relative Potency
2*R*,*S*,α*R*,*S* (technical)	0.67 (0.52–0.86)	1.0
2*S*,α*S*	0.12 (0.10–0.14)	5.6
2*S*,α*R*	11.6 (3.8–35)	0.06
2*R*,α*S*	>212	<0.003
2*R*,α*R*	>260	<0.003

their potential differential uptake, distribution, detoxification, etc., which makes it somewhat difficult to make definitive statements about their differential toxicity. One investigation was initiated to ascertain more directly the differential toxicity of the diastereomers of fenvalerate (2*S*,α*S*, 2*S*,α*R*, 2*R*,α*S*, and 2*R*,α*R*) (Bradbury et al., 1987a). It is possible that the stereoselective toxicity of diastereomers might differ in fish, compared to other species. The 2*S* pair of isomers (the 2*S*,α*S* and the 2*S*,α*R*) was shown to be 3.3 times more toxic to fathead minnow than the technical mixture (all four isomers). Table 20.1 provides a comparison of stereoselective toxicities. For reference, the 2 chiral center is the benzylic carbon in the chlorophenyl isovaleric acid portion of the molecule; the α chiral center is the cyano benzylic carbon of the phenoxybenzyl alcohol portion of this ester molecule. When the four optically pure isomers were used to treat water, each was isomerized at the α chiral center, to produce a racemic mixture in the treated water; for example, the 2S,αS isomer racemized to a mixture of the *S*,*S* and *S*,*R* isomers. This type of racemization also occurred in other protic solvents (e.g., alcohols, DMF, DMSO); therefore, it was necessary to inject the optically pure isomers intraperitoneally into the fingerling bluegill to compare directly their toxicities in that species. The resultant i.p. 48-hour LD_{50} values yielded the data shown in Table 20.1. The LD_{50} for the technical material (mixture of the four isomers) was 0.67 mg/kg; for the *S*,*S* isomer, it was 0.12 mg/kg; and for the *S*,*R* isomer, it was 11.6 mg/kg. The LD_{50} values for each of the two individual 2*R* isomers were in excess of 212 mg/kg. The pattern of relative potencies for fenvalerate isomers was determined to be approximately the same as for insects and mammals. The conclusion was drawn that stereoselective toxicity of pyrethroid isomers does not contribute to their highly potent toxicity to fish.

Secondary Mechanisms of Action

Numerous studies have shown deleterious effects of synthetic pyrethroids on Ca-ATPases and other ATPases in vertebrates and invertebrates. Because certain ATPases are involved in ion regulation, research was initiated to ascertain whether or not pyrethroids exert a toxic effect via disruption of osmoregulation. If such an osmoregulatory toxic mechanism is in play, it could be a contributing factor enhancing the toxicity of pyrethroids to fish. Acute toxicity testing of fenvalerate on juvenile bluegill was carried out at different hardnesses and salinities of the treatment water. Dyer et al. (1989) demonstrated that fenvalerate was least toxic in very soft water (6 mg/L $CaCO_3$ hardness), compared to greater hardnesses. The tissue residues were analyzed and found to be similar for all four hardnesses tested, allowing the authors to conclude that the toxicity differences were not due to variations in uptake rates. Four salinity levels were tested as well. Fenvalerate was most toxic at a salinity of 33% of seawater, which is approximately isotonic with fish blood. It was hypothesized that less enzyme induction (ATPases) may have been necessary at physiological salinity, thus requiring less fenvalerate to inhibit/disrupt a significant amount of the regulatory enzymes. Residue analysis of the fish indicated that most uptake rates were similar, but the uptake rate was significantly slower at the lowest salinity level tested (12.5% of seawater).

Another project focused on the effects of fenvalerate on regulation of specific ions in fathead minnow and bluegill (Symonik et al., 1989). The three radiotracer isotopes utilized in these studies were ^{22}Na, ^{45}Ca, and ^{36}Cl. They were used to study uptake and depuration of those important electrolytes in pyrethroid-exposed fish. Fenvalerate caused some significant perturbations in ion regulation in both species of fish, in a concentration–response manner; however, individual relationships were not easily

TABLE 20.2

Lethal Brain Concentrations of Pyrethroids

Pyrethroid	Concentration (μg/g)		
	Mouse	**Quail**	**Rainbow Trout**
cis-Cypermethrin	1.7[a]	4.0[a]	0.2[a]
Fenvalerate	1.0[b]	1.3[c]	0.16[d]
cis-Permethrin	6.0[e]	Not tested	2.0[e]
trans-Permethrin	36.4[e]	Not tested	2.0[e]

[a] Data from Edwards et al. (1985) for the 1*R*,α*S* isomer only; aqueous exposures to trout and p.o. exposures to the mouse and Japanese quail.

[b] Data from Lawrence and Casida (1982); intracerebral LC_{50} value for the 2*S*,α*S* isomer.

[c] Data from Bradbury and Coats (1982); p.o. exposure to bobwhite quail (*Colinus virginianus*).

[d] Data from Bradbury et al. (1987a); aqueous exposure.

[e] Data from Glickman and Lech (1982); i.v. and i.p. exposures.

interpreted due to limitations of the whole-body method of analysis of these three metabolic ions. There was clear evidence that the pyrethroid disrupted osmoregulation, possibly contributing, as a secondary factor, to the toxicity of this insecticide to fish.

An investigation of the effects of a pyrethroid and other toxic chemicals on behavior and morphology (development) has revealed another possible secondary toxic impact on fish. Rice et al. (1997) showed that permethrin not only was acutely toxic to juvenile medaka (at 10 μg/L and 40 μg/L) but also caused sublethal behavioral effects long before death. Specifically, permethrin produced loss of equilibrium and initial hyperactivity, followed by hypoactivity, excessive lateral flexure, and an underreactive startle response.

Fish Nervous System Sensitivity

It is feasible that the nervous system of fish is more sensitive to pyrethroids than those in mammals and birds. One possible way to investigate this possibility was to collect fish soon after death, dissect out their brains, and conduct residue analysis on them to determine the concentration required in the brain to induce mortality. Comparisons of the brain concentrations of synthetic pyrethroids at death were made from several studies that utilized fish, birds, and mammals. Table 20.2 shows the results. For *cis*-cypermethrin, the concentration required in the mouse brain for lethality is more than 8 times higher than for trout, and the concentration required in the quail brain is 20-fold higher. For fenvalerate, the amounts are 6-fold greater for mouse and 8-fold greater for quail. Higher quantities were also required for permethrin isomers. These comparative data, drawing on work from several research laboratories, indicate that the fish brain may be considerably more susceptible to pyrethroids than are mammal and bird brains. Methods developed for the study of fish neurophysiology and toxicology may help improve our understanding of their nervous system better and its susceptibility to certain classes of toxic chemicals. Noninvasive recording methods, using the startle response in Mauthner cells in larval medaka, have shown promise (Featherstone et al., 1991). Studies on the differences in types of symptoms and responses for different classes of toxic chemicals have also shown utility for the comparison of qualitative and quantitative effects on the fish nervous system (Featherstone et al., 1993).

Conclusions

Several factors probably contribute to the enhanced toxicity of synthetic pyrethroids to fish, as compared to higher vertebrate species. A summary of the factors examined here, with an assessment of their possible involvement, follows:

- More rapid uptake? Not a factor.
- More efficient distribution to nervous system? Not a factor.
- Detoxification deficiencies? Important factor.
- Slow elimination? Probably a factor.
- Differential stereoselective toxicity? Not a factor.
- Secondary modes of action? Possibly a factor.
- Nervous system sensitivity? Important factor.

Extended persistence of residues in the sediments can result from their binding to particulates in an aquatic system (Gan et al., 2005). California drainage systems are of special concern because of the high usage of pyrethroids and persistence of the residues in sediments (Oros and Werner, 2005). Foodchain exposures may result, and regeneration of bioavailable residues in water may result over time if the compounds desorb off the sediment (Amweg et al., 2005; Maund et al., 2002; Weston et al., 2004). A new emphasis on extraction and analytical methodologies (You et al., 2004) is contributing to a better understanding of pyrethroid residue persistence and bioavailability. Microbial biodegradation of pyrethroids in aquatic systems (in the sediment and the water column) has been acknowledged to play an important role in the degradability and persistence of the residues (Lee et al., 2004). Probabilistic risk assessments of pyrethroids in aquatic systems have been developed in hopes of understanding the potential adverse impacts of the insecticides on aquatic species (Solomon et al., 2001). Ethofenprox (Figure 20.2) is an example of a pyrethroid insecticide that is much less toxic to fish; notably, it is the only one without a carboxylester in the molecule. It is hoped that a better understanding of the factors that contribute to the highly potent toxicity of pyrethroids to fish will lead to the design and synthesis of safer compounds and improved methods for using them safely.

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21

Reproductive Impairment of Great Lakes Lake Trout by Dioxin-Like Chemicals

Donald E. Tillitt, Philip M. Cook, John P. Giesy, Warren Heideman, and Richard E. Peterson

CONTENTS

Introduction

The Great Lakes are the most important aquatic natural resource in North America. The Great Lakes basin is home to approximately 40 million people in the United States and an additional 20 million in Canada (Figure 21.1). The Great Lakes have been important in commerce (shipping and industry), agriculture, tourism, and recreation. The multitude of uses of these natural resources has led to a complex set of management goals that have not always been harmonized. The Great Lakes have historically supported fisheries, but direct human management and inadvertent alterations of the Great Lakes ecosystem have occurred and dramatically influenced the fisheries of the Great Lakes (Evans, 1988). Factors that have influenced Great Lakes fish populations include commercial fishing; both intentional and unintentional introduction of non-native, exotic, or alien species; stocking of different genetic strains of native fishes; and habitat loss or degradation, including decreased water quality (Eschmeyer, 1968; Eshenroder and Amatangelo, 2002; Koonce, 1990; Smith, 1971). Notable among these have been overfishing of certain populations, introductions of forage fishes and Pacific salmon, parasitism by the sea lamprey, changes in invertebrate species, and subsequent changes in nutritional status of predators (Brown et al., 2005; Eck and Wells, 1986; Miller and Holey, 1992; Muth and Busch, 1989), all of which have had adverse affects, particularly on lake trout (*Salvelinus namaycush*) populations of the Great Lakes.

Lake trout in Lake Michigan have not been naturally reproducing for over five decades. The lake trout populations in both Lakes Michigan and Ontario are maintained by stocking programs because natural reproduction of the populations is not sufficient to sustain the harvestable populations (Holey et al., 1995; Mac and Seelye, 1981b; Willford et al., 1981). There has been evidence of natural reproduction in Lake Huron (Weber and Clark, 1984), and populations have continued to reproduce naturally in Lake Superior, where reproductive success is improving (Curtis, 1990). Some studies have indicated that these recoveries are correlated with reductions in chemical contaminants observed in lake trout eggs (Cook et al., 2003; Mac and Schwartz, 1992; Mac and Seelye, 1981a,b).

Chemical contamination has resulted in degradation of key habitats, reduced habitat quality, and subsequent losses to certain fish and wildlife populations. For nearly two centuries, the Great Lakes have been the receiving waters for industrial and municipal wastes, as well as agricultural runoff. The fact that the lakes were so large resulted in the commonly held thought that it was impossible to contaminate the lakes to a sufficient degree to cause adverse environmental effects. As the populations

FIGURE 21.1 Map of the Great Lakes and drainage basin.

inhabiting the Great Lakes increased and the complexity and magnitude of the industries grew, it became apparent that it was indeed possible for exceedingly small amounts of chemicals to impact these vast ecosystems. The first such demonstration of this potential in the Great Lakes was the linkage of phosphate concentrations in the low parts per million to eutrophication, most notably in Lake Erie (Kennanek, 1971; Nurnberg, 1991; Rodgers et al., 1988). Later, the agricultural pesticide 2,2-*bis*(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was linked to eggshell thinning and adverse population effects on top avian predators such as bald eagles (Wiemeyer et al., 1975, 1993). The effects of DDT on bird populations were quite blatant and were exerted across a number of avian species. Once the demonstrative effects of DDT on bird populations had reduced with falling concentrations, more subtle effects on growth, development, and nestling survival were linked to the industrial contaminants polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), collectively referred to as halogenated aromatic hydrocarbons (HAHs) (Giesy et al., 1994; Gilbertson et al., 1991; Kubiak et al., 1989; Tillitt et al., 1992). The effects of these chemicals on avian populations in the Great Lakes, although subtle, could be tracked and investigated, because observation of the birds made it possible. The effects of chemical contamination on other components of the ecosystem, such as fish populations, proved more difficult to investigate, in part because life cycles of fish were not as easy to observe. Additionally, there have been and continue to be a large number of stressors on fish populations that are not readily quantified.

The potential for effects of contaminants on Great Lakes fishes include direct effects on the survival, growth, and reproduction of fishes and indirect effects on habitat quality. Evidence exists in support of the notion that chemicals have had both direct and indirect effects on the development of self-sustaining lake trout populations in the lower Great Lakes. The lines of evidence for direct effects of HAHs on lake trout populations in the Great Lakes are provided in this chapter. Lake trout from the Great Lakes have been and to a lesser extent continue to be contaminated with HAH mixtures. In those regions of the Great Lakes where lake trout eggs have had the greatest concentrations of HAHs, the risk of recruitment failure in lake trout populations has been substantial, and the populations have not thrived

over the last half of the 20th century (Cook et al., 2003). Trout express the aryl hydrocarbon receptor (AhR) and its dimerization partner, aryl hydrocarbon nuclear translocator (ARNT) and therefore have the capacity to respond to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related chemicals (Abnet et al., 1996; Pollenz et al., 1996). PCDDs, PCDFs, and the planar PCB congeners present in the Great Lakes ecosystem are approximate isostereomers of TCDD and are capable of causing signs of early-life-stage toxicity in trout identical to TCDD (Walker and Peterson, 1991, 1994a; Zabel et al., 1995a). Among fish species studied, lake trout are the most sensitive to TCDD-induced early-life-stage mortality, with LD_{50} values in the environmentally relevant range of 58 to 80 pg TCDD per g egg (Spitsbergen et al., 1991; Walker et al., 1991, 1994), and mixtures of the dioxin-like PCB, PCDD, and PCDF AhR agonists present in Lake Michigan lake trout eggs interact in an additive fashion to cause trout early-life-stage mortality (Walker et al., 1996; Wright and Tillitt, 1999; Zabel et al., 1995b). Taken together, these and other findings presented in this chapter support the contention that HAH contamination in the Great Lakes has led to decreased recruitment in select populations of lake trout (Cook and Burkhard, 1998; Cook et al., 1997, 2003).

Numerous adverse effects have been associated with sustained AhR activation caused by dioxin-like PCBs, PCDDs, and PCDFs. These include carcinogenesis, wasting syndrome, endocrine disruption, altered lipid metabolism, porphyria, hepatotoxicity, dermal toxicity, immunotoxicity, developmental toxicity, and reproductive toxicity (Colborn et al., 1993; Safe, 1994). In this chapter on lake trout, we focus on developmental toxicity because early life stages of vertebrates, including fish, represent the life stage most sensitive to the toxicity of PCBs, PCDDs, and PCDFs (ASTDR, 1996; Cook et al., 1993; Eisler and Belisle, 1996; Peterson et al., 1993; USEPA, 1995; Walker and Peterson, 1994a). Planar PCB congeners, PCDDs, and PCDFs are the most potent with regard to causing AhR-mediated toxicity to early life stages of trout (Walker and Peterson, 1991, 1994a; Zabel et al., 1995a) and have occurred at great enough concentrations in eggs of feral lake trout to result in early-life-stage mortality (Cook and Burkhard, 1998; Cook et al., 1997).

The goal of this chapter is to review the evidence for contaminant-related effects on lake trout populations in the Great Lakes. The history of lake trout populations is presented. The concentrations of HAHs and other persistent organic pollutants (POPs) observed in lake trout and other top predator fishes in the Great Lakes are reviewed. We also review the toxicity of HAHs to fish. Field and laboratory studies of chemical exposure in lake trout and other related species indicate that salmonines are particularly sensitive to dioxin-like chemicals. Field studies related to chemical contamination of Great Lakes salmonines are reviewed, and a case study of HAH contamination in lake trout from Lake Ontario is provided. Finally, the causative relationship between chemical pollutants and the lack of successful reproduction of Great Lakes lake trout is evaluated through a set of the criteria developed for ecoepidemiology (Fox, 1991).

Historical Aspects of the Great Lakes Lake Trout Fishery

Lake trout were historically the key top predatory fish species in the Great Lakes (Balon, 1980). Lake trout have been an important species to humans for commercial purposes, recreation, and sustenance, in the case of Native Americans. Ecologically, the lake trout represents the endemic salmonine atop the food chain, and they have served the role of "keystone predator" in the balance of the Great Lakes ecosystem. This critical role was no more apparent than in the mid-1950s, when populations of lake trout severely declined and the overabundance of forage fish resulted in massive die-offs. The dead forage fish, mainly alewives, filled the shorelines of Lake Michigan, causing problems associated with the odor, public health, and the recreational economy. They are a long-lived species of trout, with life spans of over 20 years in the lakes and 30 years in hatcheries. Lake trout are a member of the charr family and have temperature preferences of 8 to 12°C (Balon, 1980). The lake trout is slow growing and takes approximately 6 years to become reproductively active. The high fat content observed in lake trout (8 to 18%), presumably facilitating their survival in colder water temperatures, has also made them a favorite for human consumption.

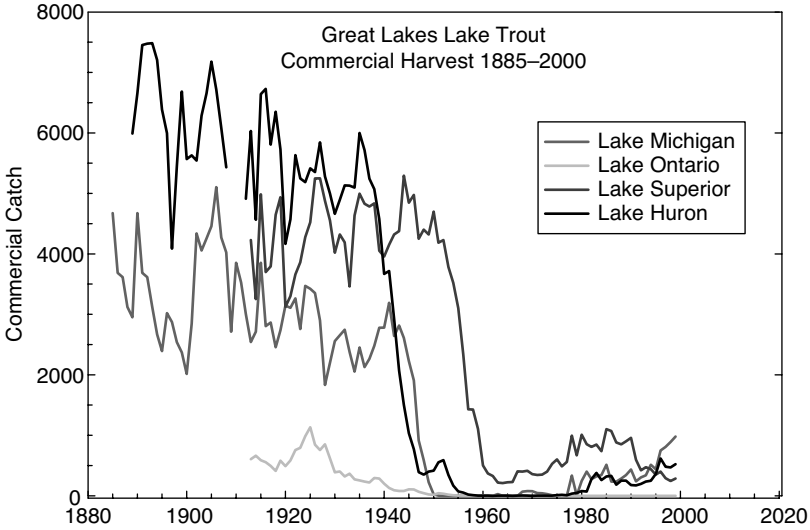


FIGURE 21.2 Great Lakes lake trout populations over the 20th century based on commercial catch per unit effort (CPUE). (Data are from Baldwin, N.A. et al., *Commercial Fish Production in the Great Lakes 1867–2000*, Great Lake Fisheries Commission, Ann Arbor, MI, 2004; http://www.glfc.org/databases/commercial/commerc.php.)

The lake trout once had self-sustaining populations in all of the Great Lakes (Figure 21.2); however, populations of adult lake trout were extirpated from the Great Lakes by the middle of the 1950s, except for the stocks in Lake Superior. Overharvesting by commercial fishermen and the accidental introduction of the sea lamprey in to the Great Lakes through the Erie Canal have been classically blamed for the demise of the populations of lake trout in the Great Lakes. Sea lampreys entered the Great Lakes in the early 1900s and have a certain proclivity for the lake trout. Sea lamprey populations grew in the Great Lakes and were considered abundant in Lakes Ontario, Erie, Huron, and Michigan by the mid-1940s (Eshenroder and Amatangelo, 2002).

Reestablishment of lake trout populations in the Great Lakes has been a major effort of the resource managers in this region. Stocking programs for the lake trout have been maintained by the U.S. Fish and Wildlife Service for the past 40 years. These programs have resulted in lake trout juveniles (100,000 annually) and fry (1.25 million annually) in the U.S. and Canadian waters of the Great Lakes (Hansen, 1999). The other major program that was initiated by resource managers around the Great Lakes to protect and restore lake trout populations was a sea lamprey control program that focused on the use of chemical control agents (specifically toxic to the larval stages of the sea lamprey) and the use of physical and electric barriers to stop the migration of adult sea lamprey up rivers to their spawning areas (Jude et al., 1981; Marsden et al., 1988). The lampricide that had the greatest use was 3-trifluoromethyl-4-nitrophenol (TFM), which was sprayed in the shallow, backwater, spawning grounds of rivers known to have sea lamprey spawning runs. TFM is toxic to the larval (amneocete) stage after the sea lamprey emerges from the bottom sediments, while resident fish are not affected (Applegate et al., 1961). Other rivers were fitted with mechanical weirs capable of stopping the adult sea lamprey from migrating upstream to spawn. Both of these programs continue in the Great Lakes today. These extensive programs have been largely successful in reestablishing adult populations of lake trout in all of the Great Lakes where they had previously thrived.

Although populations of lake trout were reestablished, there was little or no evidence of natural reproduction in certain lakes of the Great Lakes. The years of stocking fish, control of lamprey populations, and ban on commercial fishing were successful in the restoration of adult populations of lake trout; however, even with ample numbers of lake trout of spawning age that produced viable gametes, signs of natural reproduction in this species in the lower Great Lakes are meager. No signs of natural reproduction have been observed in Lake Michigan since the stocking programs began over 40 years

ago. Natural reproduction of lake trout was observed in northern Lake Huron in the early 1990s (Hansen et al., 1995; Holey et al., 1995; Johnson and VanAmberg, 1995), and Lake Superior has always maintained a natural, self-sustaining stock of lake trout (Bronte, 1993; Holey et al., 1995). Lake Ontario has only had evidence of a small amount of natural reproduction over the past two decades (Marsden and Krueger, 1991; Marsden et al., 1988). Thus, even though adult populations of lake trout exist, natural recruitment of young of the year lake trout is nonexistent (Lake Michigan), slight (Lake Ontario), or not enough to maintain self-sustaining populations (Lake Huron) in the lower Great Lakes, which historically had thriving populations of lake trout.

Chemical Contamination of the Great Lakes and Lake Trout

Concentrations of chlorinated hydrocarbons, as well as some metals, have been monitored in the tissues of fishes of the Great Lakes since the mid-1970s (Allan et al., 1991; Clark et al., 1984; DeVault, 1985; Fitchko 1986; Hitchin et al., 1993; Veith, 1975; Willford, 1980). Over this period, literally hundreds of studies have measured the concentrations of chlorinated hydrocarbons in fishes from the Great Lakes (Baumann and Whittle, 1988; DeVault et al., 1986; Hickey et al., 2006; Rodgers et al., 1993). Numerous reports on monitoring programs conducted by state, provincial, and federal laboratories and annual reports have been published; thus, extensive information is available regarding the status and trends of concentrations of contaminants in fishes of the Great Lakes (Baumann and Whittle, 1988; Fitchko, 1986; Hickey et al., 2006). The focus of most monitoring efforts has been on the high-use, persistent, bioaccumulative chemicals such as organochlorine pesticides, PCBs, and mercury (Tillitt et al., 1998). Monitoring programs have been designed to determine trends for known pollutants, rather than identify new types of compounds. More recently monitoring efforts in the Great Lakes have included chemicals other than the classical organochlorine pesticides and PCBs (Giesy et al., 2006); however, trend data are not available for many of these more modern chemical contaminants, unless they are persistent enough to allow screening of archived samples (Zhu and Hites, 2004).

Concentrations of hydrophobic contaminants in fishes are dependent on a number of parameters, such as species, age, gender, season, food sources, and collection location, in addition to chemical-source-related factors. In general, the larger, older, fish, including lake trout, have greater concentrations of the HAHs. Also, fish with greater lipid content and those at the top of the food chain tend to have the greatest concentrations of HAHs. Thus, fish, such as lake trout and brown trout tend to have the greater concentrations of HAHs and organochlorine pesticides as compared to other top predators and species lower on the trophic chain. Walleye, for example, are the top predator in Lake Erie, but generally concentrations of these contaminants in walleye do not reach the same concentrations as measured in salmonines due to the smaller lipid content in walleye, their lower trophic status, and the reduced longevity of walleye compared to lake trout (DeVault et al., 1986; Hickey et al., 2006).

The greatest concentrations of HAHs and organochlorine pesticides in Great Lakes fish tend to be found in fish from Lakes Ontario and Michigan (Allan et al., 1991; Fitchko, 1986; Bauman and Whittle, 1988). This has been true over the past 40 years and remains true today. Fish from Lake Ontario contain the greatest concentrations of the insecticides mirex, DDT, and dieldrin, while the concentrations of toxaphene are greatest in fishes from Lake Superior (Allan et al., 1991; Baumann and Whittle, 1988; Hickey et al., 2006). Concentrations of persistent chlorinated hydrocarbons have historically been least in fishes from Lake Superior and Lake Huron (Armstrong and Lutz, 1977a;b; Baumann and Whittle, 1988; Simmons, 1984). The trends of most of these compounds in fish from the Great Lakes have been declining until recently (DeVault et al., 1996; Hickey et al., 2006). Currently, most of these contaminant concentrations in Great Lakes fish have reached an apparent steady state or only slightly decreasing concentrations (Hickey et al., 2006). The greatest rates of declines in fish contaminant concentrations have occurred in Lake Ontario and Lake Michigan (Baumann and Whittle, 1988; Maack and Sonzogni, 1988). These lakes have experienced the greatest decreases largely due to the fact they had the greatest contamination from industrial point-sources or agricultural activities; yet, current concentrations of PCBs and DDT in fish from Lakes Michigan and Ontario remain the greatest in the Great Lakes, due to the

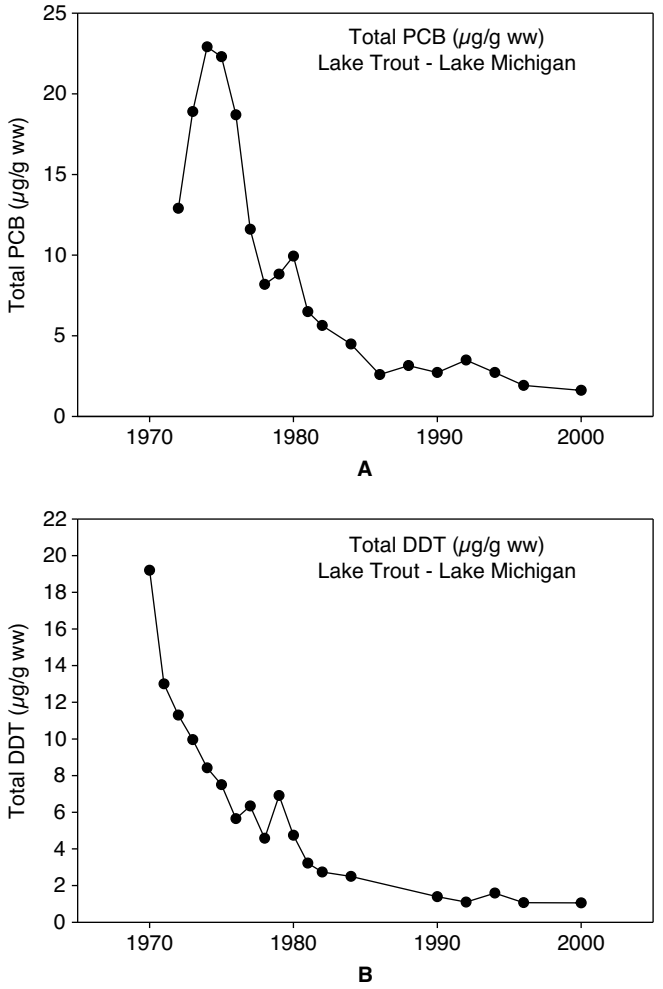


FIGURE 21.3 Concentrations (μg/g ww) of (A) total polychlorinated biphenyls (PCBs) and (B) 2,2-*bis*(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) in lake trout from Lake Michigan, 1970 to 2000. (Data are from DeVault, D.S. et al., *J. Great Lakes Res*., 22, 884–895, 1996; Hickey, J.P. et al., *Arch. Environ. Contam. Toxicol*., 50, 97–110, 2006.)

slow release of these contaminants from sediments in point-source river beds. Concentrations of both PCBs and DDT in lake trout from Lake Michigan have declined greatly, from a maximum of greater than 20 mg/kg in 1973 to a current average of approximately 1.0 mg/kg (Figure 21.3). The rate of decrease in concentrations of PCBs during the last two decades of the 20th century was greatest in fish from Lake Michigan compared to the other Great Lakes (Hickey et al., 2006). Concentrations of both PCBs and DDT in lake trout from Lake Superior have always been the least of all of the Great Lakes (Baumann and Whittle, 1988).

The concentrations of AhR agonists in the Great Lakes food web have declined over the past four decades; however, with the exception of PCB concentrations, documentation of these declines in Great Lakes fish is not as complete as that of organochlorine pesticides. The information on temporal trends in PCDD and PCDF concentrations in Great Lakes fish is limited. A single monitoring program that has sustained measurements of these analytes in Great Lakes fish does not exist; however, based on the various studies that have been reported on the concentrations of PCDDs and PCDFs in fish, declines appear to follow those of organochlorine pesticides and PCBs. DeVault and coworkers (1989) reported concentrations of HAHs in lake trout collected in 1984 from Lakes Michigan, Ontario, Huron, and Superior and in walleye collected from Lake Erie that same year. The concentrations of PCDDs and

PCDFs were greatest in fish collected in Lake Ontario. The potencies of PCDD, PCDF, and planar PCBs are summed by the use of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQs) (van den Berg et al., 1998). TEQs calculated based on fish-specific TCDD toxicity equivalency factors (TEFs) were 70, 30 (±13), 28, 7, and 6 pg/g in fish collected from Lakes Ontario, Michigan, Huron, Erie, and Superior, respectively (DeVault et al., 1989). Slightly greater concentrations of PCDDs and PCDFs were reported in Great Lakes fish collected earlier (Stalling et al., 1983). These data do not allow direct comparisons of TEQs, but the reported concentrations are consistent with the findings of DeVault and coworkers (1989). Concentrations of PCDDs and PCDFs in lake trout collected from Lake Michigan were less in 1988 (12.7 pg/g) (Wright and Tillitt, 1997), as compared with the earlier collections and consistent with a decline over time. The most complete evaluation of temporal trends of PCDDs and PCDFs in lake trout has come from predictions based on analysis of dated sediment cores (Cook et al., 2003). Estimated concentrations of TEQs in lake trout from Lake Ontario were maximum (250 to 275 pg/g) in the mid-1960s and declined exponentially from the 1970s to the 1990s. A detailed analysis of this data and the subsequent risk analysis are presented later in this chapter.

The current trends in concentrations of chlorinated hydrocarbons are not expected to decrease as much as previous years; instead, they are expected to slowly decline as the number of point sources continues to decrease. In the case of PCBs, although the concentrations entering the lakes from point sources is now less than at the peak of releases in the late 1960s, large stores of PCBs are still present in the terrestrial environment and in contaminated sediments; thus, PCBs are still entering the lakes. Nevertheless, concentrations of PCBs in fish tissues have decreased by a factor of approximately 25 since maximum concentrations were reached in the lower Great Lakes in the mid-1970s. Although there are differences among species and locations, in general the trends for persistent halogenated hydrocarbons in Great Lakes fish during the last decade are either slightly decreasing or fluctuating around an apparent steady state (Hickey et al., 2006; Tillitt et al., 1998). Most POPs have been banned in the industrialized countries of North America and Europe from manufacture and continued new use; however, long-range, atmospheric transport is also responsible for new releases of POPs into the Great Lakes (Baker, 1997). The northern latitudes of the lakes, along with the large surface area, make atmospheric deposition an important source of contaminant loading. It is difficult with the current datasets to ascertain whether concentrations of chlorinated hydrocarbons have attained a new equilibrium or whether the rates of decline are slowing. Regardless, changes in concentration are difficult to distinguish on an annual basis, and it appears that current concentrations of HAHs and organochlorine pesticides will not decline greatly over the next decade.

Concentrations of mercury in lake trout were measured as part of the National Contaminants Biomonitoring Program (NCBP) of the U.S. Department of Interior (Schmitt et al., 1985, 1990). Other monitoring programs, such as those of the U.S. Environmental Protection Agency (USEPA), did not include mercury in their list of targeted analytes until this century. The concentrations of mercury in lake trout from the Great Lakes remained fairly constant during the period from the mid-1970s until the mid-1980s. Concentrations of mercury were 0.15 to 0.33 µg/g in Lake Superior, 0.12 to 0.17 µg/g in Lake Michigan, 0.15 to 0.29 µg/g in Lake Huron, 0.12-0.17 µg/g in Lake Erie, and 0.30 to 0.40 µg/g in Lake Ontario fish (Schmitt et al., 1990). The Canadian Department of Fisheries and Oceans (DFO) has maintained extensive contaminant monitoring efforts in the Great Lakes for the past three decades and has monitored mercury in the aquatic ecosystem through measurements in rainbow smelt (*Osmerus mordax*). From 1981 to 2002, concentrations of mercury in rainbow smelt from the Great Lakes ranged from 0.02 to 0.1 µg/g in Lake Superior, 0.05 to 0.07 µg/g in Lake Huron, 0.02 to 0.04 µg/g in Lake Erie, and 0.04 to 0.08 µg/g in Lake Ontario (Great Lakes Binational Toxics Strategy, http://www.epa.gov/glnpo/bns/dioxin/index.html).

Aryl-Hydrocarbon-Receptor-Mediated Toxicity in Fish

Biological responses elicited by dioxin-like PCBs, PCDDs, and PCDFs are triggered by binding to a cellular protein: the aryl hydrocarbon receptor (AhR). This protein is highly conserved in vertebrates and is a member of the PAS family (*per–arnt–sim* gene family) of basic helix–loop–helix regulatory proteins. Its role as a ligand-activated transcription enhancer is well understood in the context of induction

of biotransformation enzymes such as cytochrome P4501A1. It is an orphan receptor in that chemicals endogenous to the body that bind to and activate the AhR under physiological conditions exist and are beginning to be isolated from animal tissue and identified (Henry et al., 2006; Song et al., 2002). The AhR may have an extranuclear function in regulating signal transduction via calcium signaling and tyrosine phosphorylation (Enan and Matsumura et al., 1996), but this pathway is less understood. PCB, PCDD, and PCDF congeners that bind to and activate the AhR are considered to produce toxicity by an AhR-mediated mechanism (Poland and Knutson, 1982). The structure–activity relationships for PCDD, PCDF, and PCB congeners among fish and mammals are similar with respect to producing TCDD-like responses, with the notable exception that the mono-*ortho*-substituted PCBs are far weaker inducers of cytochrome P4501A activity in fish (Gooch et al., 1989) and are inactive in causing early-life-stage mortality in rainbow trout (Walker and Peterson, 1991; Zabel et al., 1995a).

Presence of AhR and ARNT in Fish and the AhR Signaling Pathway

Fish express both the AhR and its dimerization partner the AhR nuclear translocator (ARNT); however, fish differ from mammals in possessing multiple (two to six) AhRs (Hahn et al., 2006). These AhRs have been detected in several bony fishes (Osteichthyes) including rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), Atlantic killifish (*Fundulus heteroclitus*), winter flounder (*Pleuronectes americanus*), Atlantic tomcod (*Microgadus tomcod*), scup (*Stenotomus chrysops*), zebrafish (*Danio rerio*), and two cartilaginous fishes (Chondrichthyes), the smooth dogfish (*Mustelis canis*) and spiny dogfish (*Squalus acanthias*) (Hahn and Karchner, 1995; Hahn et al., 1992, 1997; Hansson et al., 2003, 2004; Roy and Wirgin, 1997; Stegeman and Hahn, 1994). The presence, forms, and functions of the AhR in fishes have been recently reviewed (Hahn, 2002; Hahn and Hestermann, 2008; Hahn et al., 2006). Two ARNT proteins that bind the AhR have been detected in rainbow trout (Pollenz et al., 1996) as well as in zebrafish (Tanguay et al., 2000) and killifish (Powell et al., 1999). Other members of the PAS superfamily of transcription factors include PER1, 2, and 3; SIM1 and SIM2; AHRR, ARNT2; hypoxia inducible factors (HIF-1α, -2α, 3α); SRC-1, 2, and 3; CLOCK1 and CLOCK2; NPAS1 and NPAS3; BMAL1 and BMAL2; and NXF (see Table 1 of Hahn et al., 2006, for a complete list). ARNT forms heterodimers with HIFs, PERs, SIMs, and AhRR (Hogenesch et al., 1997; Mimura et al., 1999; Tian et al., 1997). The presence of multiple binding partners of ARNT suggests that these PAS proteins may compete with each other for binding to ARNT. In this context, it is possible that formation of the TCDD/AhR/ARNT complex could deplete the available ARNT from its physiological functions. Activation of the AhR signaling pathway occurs when TCDD-like PCB, PCDD, and PCDF AhR agonists enter the cell and bind to the cytosolic AhR. Upon TCDD binding and translocation of the liganded AhR to the nucleus, two heat shock proteins (HSP90) that stabilize the cytosolic AhR for ligand binding dissociate from the AhR along with another protein, AIP (Whitlock, 1993). In the nucleus, the activated AhR dimerizes with ARNT (Pollenz et al., 1994; Probst et al., 1993). The ligand-bound AhR/ARNT complex recognizes and binds dioxin response elements (DREs) found in promoters of responsive genes to alter gene expression. This primary event is considered to eventually have led to the range of biological responses produced by exposure to TCDD-like AhR agonists (Sutter et al., 1991; Whitlock, 1993). Responsive genes include detoxification enzymes such as cytochrome P4501A1 and genes involved in differentiation and proliferation (Sutter and Greenlee, 1992; Whitlock, 1993).

Functional Mechanisms of Dioxin Toxicity Are Revealed in Fish

In the last decade zebrafish (*Danio rerio*) have become the prototype species for investigating mechanisms of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) developmental toxicity and aryl hydrocarbon receptor signaling in fish. In contrast to lake trout, which are among the species most sensitive to TCDD developmental toxicity, zebrafish are among the least sensitive. Nevertheless, if zebrafish larvae are exposed to a sufficiently high concentration of TCDD, essentially the same endpoints of toxicity are observed, culminating in mortality associated with blue sac syndrome. The difference in potency of TCDD for eliciting larval toxicity in freshwater, estuarine, and marine fish species is poorly understood. Future research on AhR signaling in trout and zebrafish may shed light on the biological basis for this difference.

The zebrafish is ideally positioned phylogenetically to make it a useful model for investigating TCDD developmental toxicity. Zebrafish, as vertebrates, are closely related to humans, so results on TCDD toxicity in zebrafish embryos and larvae are of human health relevance. Zebrafish, as fish, are useful for identifying adverse effects of TCDD exposure in fish and for functionally characterizing the AhR signaling pathway in fish that mediates TCDD toxicity. Like trout, the zebrafish is an oviparous species that has external fertilization and development; however, the time required for zebrafish to undergo early development is much shorter than lake trout and rainbow trout, making the zebrafish ideal for hypothesis testing in research on developmental toxicity. Also, unlike trout, the zebrafish embryo is transparent, which permits easy detection of morphological alterations caused by TCDD. In addition to their short generation time, zebrafish exhibit rapid growth and high egg yield and their genetics and developmental biology are well known. The sequenced zebrafish genome is now being assembled and annotated, and many molecular approaches exist to study gene function in zebrafish, including the production of transgenic and mutant fish and the ability to selectively block the expression of certain genes in zebrafish embryos using morpholino oligonucleotide (MO) knock-down technology (Nasevicius and Ekker, 2000; Udvadia and Linney, 2003). Thus, the zebrafish model has great appeal for developmental toxicologists. Among all environmental contaminants evaluated for developmental toxicity in zebrafish (Carney et al., 2006a,b; Hill et al., 2005; Spitsbergen and Kent, 2003; Tanguay et al., 2003), TCDD is the most extensively studied. Target organs of TCDD developmental toxicity have been identified and components of the AhR signaling pathway functionally characterized in zebrafish. Progress has been made in understanding how hyperactivation of the AhR pathway by TCDD leads to developmental toxicity in zebrafish by a CYP1A-independent mechanism.

AhR Signaling in Fish

The AhR is a member of the basic helix–loop–helix PAS family of transcription factors (Huang et al., 1993). Proteins in the α-class, such as AhR and hypoxia-inducible factor-1α (HIF-1α), function as sensor proteins. Once activated, α-class proteins dimerize with β-class proteins, such as ARNT1 and ARNT2, and the heterodimer (i.e., AhR/ARNT) binds specific DNA sequences termed AhR response elements (AhREs), leading to altered gene expression. The AhR has three functions: (1) *adaptation signaling*, leading to upregulation of xenobiotic metabolizing enzymes; (2) *toxic signaling*, causing adverse effects from persistent, high-affinity ligands such as TCDD; and (3) *developmental signaling*, resulting in normal development of certain organs and tissues (Denison and Nagy, 2003; Fernandez-Salguero et al., 1996; Gu et al., 2000; Mimura et al., 1997; Peters et al., 1999; Walisser et al., 2004). All three types of signaling require sequential ligand activation of the AhR, translocation of the liganded receptor to the nucleus and heterodimerization of AhR with ARNT.

To establish zebrafish as a model for investigating TCDD developmental toxicity in fish, it was essential that components of the AhR signaling pathway in zebrafish (Figure 21.4) be identified and functionally characterized (Andreasen et al., 2002a; Carney et al., 2004, 2006a; Evans et al., 2005; Prasch et al., 2006; Tanguay et al., 1999, 2000; Zodrow et al., 2001). Zebrafish express three forms of AhR: zfAHR1A, zfAHR1B, and zfAHR2 (Andreasen et al., 2002a; Karchner et al., 2005; Tanguay et al., 1999). The three zfAHRs exist as a result of gene duplications that occurred during vertebrate evolution as well as a genome duplication specific to the fish lineage. Multiple *ahr* genes are also found in other fish species (Hahn et al., 1997, 2006). Likewise, there are two *arnt* genes in zebrafish. ZfARNT2, which exists as multiple splice variants, was the first zebrafish ARNT identified as a possible dimerization partner for zfAHRs (Hsu et al., 2001; Tanguay et al., 2000; Wang et al., 2000). Later, a second *arnt* gene, *zfarnt1*, was identified in zebrafish and the zfARNT1 protein was determined to be the dimerization partner for zfAHR2 involved in mediating TCDD developmental toxicity (Antkiewicz et al., 2006; Prasch et al., 2006). Additional components of the zebrafish AhR signaling pathway include two AhR repressor genes, *zfahrr1* and *zfahrr2* (Evans et al., 2005). Zebrafish embryo exposure to TCDD upregulated *in vivo* expression of zfAHRs and repressed *in vitro* transactivation of the AhR/ARNT heterodimer (Evans et al., 2005). The *in vivo* role of the zfAHRs in modulating TCDD developmental toxicity in zebrafish, however, has yet to be established. An AhR interacting protein (AIP) has been identified in zebrafish (Zodrow et al., 2001), and its role in zfAHR signaling is being investigated.

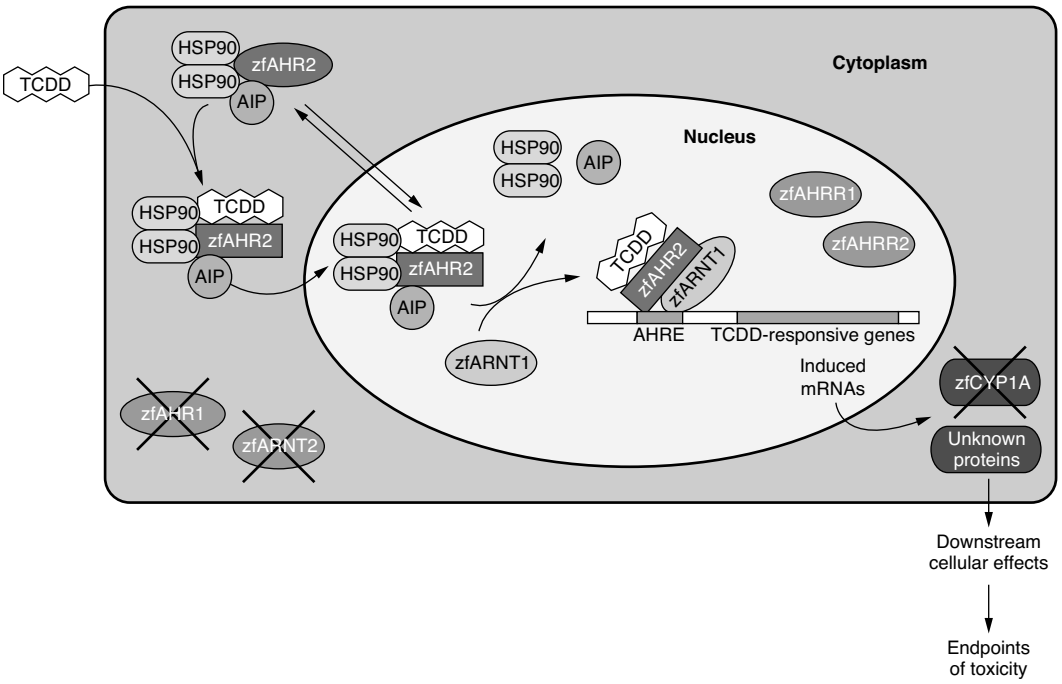


FIGURE 21.4 Aryl hydrocarbon receptor (AhR) signaling pathway in zebrafish. The AhR and ARNT isoforms marked with an "X" are not in the functional pathway of AhR-related toxicity in zebrafish, as is the case with zfCYP1A.

Endpoints of TCDD Developmental Toxicity in Fish

Like other fish species, zebrafish embryos exposed to TCDD at the blastula stage develop normally through gastrulation and primary organogenesis. TCDD toxicity is not manifested until later stages of development when morphogenesis of primary organ systems and embryo growth occur. For zebrafish, the early-life-stage period when endpoints of developmental toxicity are first manifested occurs 48 to 120 hours post-fertilization (hpf). If developing zebrafish are exposed to TCDD, prior to or during this time, TCDD toxicity is manifested 48 to 120 hpf by cardiovascular dysfunction culminating in heart malformation, peripheral ischemia and eventually heart failure, impaired osmoregulation culminating in edema, impaired erythropoiesis leading to anemia, hemorrhage, reduced jaw growth and jaw malformation, altered brain morphology, body growth retardation, uninflated swim bladder, and, beginning around 144 hpf, mortality associated with blue sac syndrome (Antkiewicz et al., 2005; Belair et al., 2001; Bello et al., 2004; Carney et al., 2006b; Dong et al., 2002; Henry et al., 1997; Hill et al., 2003, 2004a,b; Teraoka et al., 2002). The gross morphological manifestations of TCDD developmental toxicity in zebrafish are edema, cardiovascular toxicity, anemia, impaired chondrogenesis, and neurotoxicity.

Edema

The hallmark response to TCDD in the zebrafish embryo is the accumulation of edema fluid in the pericardium beginning at 72 hpf and in the yolk sac beginning at 96 hpf (Belair et al., 2001; Dong et al., 2002; Henry et al., 1997; Hill et al., 2004a). The edema may be caused by TCDD-induced disruption of osmoregulatory or circulatory function. In the zebrafish embryo, osmoregulation is carried out by the pronephric kidney and skin (Drummond et al., 1998; Hagedorn et al., 1998; Rombough, 2002). Also, due to the close association between osmoregulatory function of the skin and kidney and circulatory function of the heart and vasculature, edema may be secondary to cardiovascular dysfunction. The gills are less likely to be involved because their osmoregulatory function does not occur until later, after the critical period for TCDD developmental toxicity has passed. Although glomerular filtration is largely unaffected by TCDD prior to edema (Hill et al., 2004a), peripheral blood flow is reduced (Carney et al.,

2005). Rearing TCDD-treated embryos in an isosmotic mannitol solution prevents edema but does not prevent cardiac toxicity (Antkiewicz et al., 2005; Hill et al., 2004a). TCDD also increases vascular permeability in the dorsal midbrain of the zebrafish embryo (Dong et al., 2004). Thus, several factors may lead to edema in TCDD exposed zebrafish larvae, including heart failure and an increase in permeability of the skin and/or vasculature.

Cardiovascular Toxicity

In zebrafish embryos exposed to TCDD, both the heart and vasculature are target organs, and the ensuing cardiovascular toxicity is characterized by ischemia and impaired growth and development of the heart and vasculature culminating in heart failure and mortality (Antkiewicz et al., 2005; Bello et al., 2004; Carney et al., 2006b; Henry et al., 1997; Teraoka et al., 2002). Embryos of zebrafish exposed to a lethal concentration of TCDD shortly after fertilization initially develop a normal circulation and a functional beating heart; however, at 48 hpf the first endpoints of cardiac toxicity emerge as subtle changes in heart morphology followed by reduced blood flow in certain vascular beds and a slight accumulation of edema fluid in the pericardial sac. As TCDD developmental toxicity evolves over the next 72 hours, the endpoints of cardiovascular toxicity become strikingly more evident. At 120 hpf, failure of an abnormally small malformed heart, associated with virtually no peripheral blood flow, severe pericardial edema, heart malformation, and ventricular standstill, occurs (Antkiewicz et al., 2005; Belair et al., 2001; Carney et al., 2006b; Prasch et al., 2003). Until recently, the earliest occurring adverse cardiovascular response to TCDD observed in the zebrafish embryo was decreased blood flow in certain peripheral vascular beds; for example, a transient decrease in blood flow was detected in the mesencephalic vein of the dorsal midbrain at 50 hpf (Dong et al., 2002), but decreases in blood flow in other major vascular beds were not seen until about 72 hpf (Belair et al., 2001; Prasch et al., 2003; Teraoka et al., 2002). This characteristic, later occurring, profound peripheral ischemia that is caused by TCDD exposure in zebrafish larvae is secondary to decreased cardiac output. The drop in cardiac output is caused by a decrease in stroke volume; reduced heart rate does not play a role (Antkiewiez et al., 2005; Carney et al., 2006b). Reductions in peripheral blood flow precede reductions in heart rate by at least 24 hours (Antkiewicz et al., 2005; Henry et al., 1997). The number of myocytes in the heart is reduced by about 20% in TCDD-treated embryos at 48 hpf, and the percent reduction in cardiac myocyte number at 96 hpf is even greater (24%) (Antkiewicz et al., 2005). This decrease in cardiomyocyte number causes the overall size of the heart to be smaller and stroke volume to be less, leading to the term *small heart syndrome* (Antkiewicz et al., 2005, 2006; Carney et al., 2006b).

Anemia

The formation of red blood cells is reduced by TCDD exposure in zebrafish. The zebrafish embryo proceeds through early hematopoiesis 18 to 96 hpf (Davidson et al., 2004); however, TCDD blocks the switch from primitive to definitive erythropoiesis, resulting in larvae that lack circulating definitive erythrocytes (Belair et al., 2001). The mechanism by which TCDD disrupts this developmental erythropoietic process is unknown.

Impaired Chondrogenesis

Exposure of the zebrafish embryo to TCDD also impairs development of cartilage structures in the lower and upper jaw and the cranium (Henry et al., 1997; Hill et al., 2004b; Teraoka et al., 2002). More specifically, TCDD inhibits jaw cartilage growth and orientation but not initial formation of the jaw cartilage structures. All cartilaginous structures are present in the jaw of the TCDD-treated zebrafish embryo, but they are reduced in size and altered in shape (Hill et al., 2004b; Teraoka et al., 2002). Because the adverse effects of TCDD on jaw development precede reductions in jaw blood flow, a primary effect of TCDD is to decrease jaw cartilage growth (Carney et al., 2005; Teraoka et al., 2002). Furthermore, the decrease in jaw cartilage length in TCDD-treated embryos is greater than the reduction in body length, suggesting it is unlikely for the inhibitory effect of TCDD on jaw growth to be secondary to stunted growth of the whole fish (Teraoka et al., 2002; Hill et al., 2004b). A final point, the inhibitory effect of TCDD on cartilage growth in the zebrafish differs from its disruptive effects on osmoregulation,

cardiovascular function, and hematopoiesis. This is because there is not a critical developmental window for eliciting the inhibitory effect of TCDD on cartilage growth like there is for endpoints of developmental toxicity such as the smaller malformed heart, edema, or anemia (Belair et al., 2001; Carney et al., 2005; Heideman et al., 2001). This difference is illustrated by the ability of TCDD to stunt the growth of lower jaw cartilage, not only in zebrafish exposed to TCDD as embryos and larvae but also in zebrafish exposed to TCDD as juveniles and adults.

Neurotoxicity

The developing zebrafish brain is adversely affected by TCDD. At approximately 50 hpf, a small increase in apoptosis is observed in the dorsal midbrain (Dong et al., 2001, 2002). In addition, more prominent effects are seen at later stages of brain development; for example, TCDD causes a 29% reduction in brain volume at 168 hpf that is associated with a decrease in the total number of neurons in the brain (Hill et al., 2003). It is unclear if TCDD affects the zebrafish brain directly or if the decrease in number of neurons is secondary to a decrease in brain blood flow. In support of the latter, an increase in apoptosis in the midbrain of TCDD-exposed zebrafish embryos has been associated with decreased blood flow to the dorsal midbrain region (Dong et al., 2002).

Female Reproductive Toxicity of TCDD in Fish

Although much has been learned regarding the impacts of TCDD on early embryonic development of zebrafish, relatively little is understood about the impacts of TCDD on the reproductive system of adult female zebrafish. Wannamacher et al. (1992) showed that acute dietary exposure of 5 to 20 ng TCDD induces overt toxicity associated with a dose-dependent reduction in egg production and complete suppression of spawning activity, corresponding with arrested gonadal development and oocyte atresia. Unfortunately, the small sample size of this study made reproductive toxicity difficult to evaluate, and a dose–response relationship for TCDD-induced reproductive toxicity could not be determined because levels of TCDD were not measured in females or eggs. More recently, King Heiden et al. (2005, 2006) found that the reproductive success of female zebrafish was impaired when exposed to concentrations of TCDD that do not induce acute toxicity. They demonstrated that sublethal concentrations of TCDD are capable of modulating reproductive success of female zebrafish even when spawning activity and overall egg production are not decreased and that subtle physiological changes produced by TCDD can lead to attenuated follicular development and ovarian steroidogenesis. Reproductive effort measured by the ovarian somatic index is significantly reduced following the accumulation of 0.6 ng TCDD per g female (King Heiden et al., 2005); following an estimated accumulation of 4.0 ng TCDD per g female, egg production and spawning success are decreased (King Heiden et al., 2006). Following the accumulation of 3.0 ng TCDD per g female (0.3 ng TCDD per g egg), maternal transfer reduces offspring survival, while maternal transfer of as little as 0.094 ng TCDD per g egg induces the typical signs of larval toxicity (King Heiden et al., 2005). Even when overall egg production is not impacted, subtle physiological changes induced by TCDD can lead to altered follicular development and decreased serum 17β-estradiol and vitellogenin concentrations (King Heiden et al., 2006). Histopathological analyses suggest that, although liver toxicity may contribute to observed impacts on follicular development and vitellogenesis, reproductive toxicity of TCDD likely results from direct action at the ovary by inhibiting follicular development, in addition to inducing follicular atresia. Taken together, these findings support the hypothesis that maternal transfer of low concentrations of TCDD can have profound effects on offspring health and survival and potentially impact recruitment in natural populations and that long-term exposure to very low concentrations of TCDD could potentially impact fecundity.

The mechanisms by which TCDD induces these reproductive alterations have not been fully characterized. In an effort to identify the transcriptional changes that precede observed ovarian toxicities, King Heiden et al. (2007) used quantitative reverse-transcription polymerase chain reaction (RT–PCR) to assess the effect of TCDD on the expression of several candidate genes important in the regulation of follicular development and steroidogenesis. Additionally, global changes in gene expression in the ovary caused by TCDD exposure were identified using microarray analysis. Their data suggest that suppression

of several genes important for the regulation of follicular development (e.g., *lhr*, *fshr*, and all three estrogen receptors) and estrogen biosynthesis (e.g., *cyp19a1a*) likely contribute to observed impacts of TCDD on follicular development and reduced serum estradiol concentrations. Global changes in gene expression demonstrate that TCDD impacts several integrated cellular pathways, resulting in alterations in the expression of genes important for glucose and lipid metabolism, regulation of transcription, and immune function. Their data also suggest that, although AhRE-mediated changes in gene expression likely contribute to TCDD-induced ovarian toxicity, alterations in gene expression may be downstream from AhR activation and AhRE-regulated transcription. Further, interference with estrogen-regulated signal transduction by AhR signaling is likely a key component of the ovarian toxicity of TCDD.

ZfAHR2 Is Required for TCDD Developmental Toxicity in Zebrafish

It is not clear how activation of AhR signaling by TCDD produces such diverse biological responses in zebrafish larvae; however, a variety of factors are probably involved, including AhR agonist potency, duration of AhR activation, organism life stage, and differential responsiveness of cells and tissues to AhR activation by TCDD (Walisser et al., 2004). *In vitro* assays suggest that zfAHR2 is the likely AhR isoform that mediates responses to TCDD in zebrafish. Radioligand-binding assays demonstrate that zfAHR2 specifically binds TCDD (Andreasen et al., 2002a), and exposure to the AhR ligand β-naphthoflavone causes zfAHR2 to translocate to the nucleus (Wentworth et al., 2004), where it can dimerize with ARNT. ZfAHR2 and one form of zfARNT2 (zfARNT2b) have been shown to form a functional heterodimer *in vitro* that specifically binds AhRE sequences in gel mobility shift assays and induces AhRE-driven reporter gene activity in COS-7 cells exposed to TCDD (Tanguay et al., 2000). In contrast, zfAHR1 is unable to bind TCDD in radioligand-binding studies and only weakly interacts with AhRE sequences or promotes AhRE-driven transcription when expressed with ARNT2 proteins (Andreasen et al., 2002a). To determine if zfAHR2 mediates TCDD developmental toxicity, antisense morpholino oligonucleotides (MOs) were used to knock down levels of zfAHR2 protein expression in zebrafish embryos, creating *zfahr2* morphants, and the morphants were subsequently exposed to TCDD (Antkiewicz et al., 2006; Bello et al., 2004; Carney et al., 2004; Dong et al., 2004; Hill et al., 2004a; Prasch et al., 2003; Teraoka et al., 2003). The zfAHR2 morpholino results convincingly demonstrate that zfAHR2 is required for mediating the hallmark endpoints of TCDD toxicity in developing zebrafish. *Zfahr2* morphants show decreased levels of zfCYP1A induction after TCDD exposure, demonstrating that induction of zfCYP1A requires zfAHR2 (Carney et al., 2004; Dong et al., 2004; Prasch et al., 2003; Teraoka et al., 2003). *Zfahr2* morphants also show protection against certain cardiovascular endpoints of TCDD developmental toxicity, including reduced peripheral blood flow, reduced stroke volume, decreased cardiac output, reduced number of cardiomyocytes in the heart, increased incidence of ventricular standstill, impaired common cardinal vein migration, pericardial edema, and impaired erythrocyte maturation (Antkiewicz et al., 2006; Bello et al., 2004; Carney et al., 2004; Prasch et al., 2003; Teraoka et al., 2003). The *zfahr*2-MO also provides protection against the TCDD-induced increase in apoptosis in the dorsal midbrain (Dong et al., 2004) and partial protection against reductions in lower jaw growth (Prasch et al., 2003). A possible reason for why the TCDD-induced impairment in lower jaw growth is only partially protected by treatment with *zfahr*2-MO is because impaired jaw growth can still be elicited by the TCDD that remains in the fish after the effect of the *zfahr2*-MO has worn off and zfAHR2 levels return to normal (Prasch et al., 2003). In contrast to the findings with zfAHR2, embryos injected with a morpholino targeted against zfAHR1A do not show significant protection against any of the overt endpoints of TCDD developmental toxicity (Carney, unpublished results); thus, zfAHR2 is the AhR isoform that mediates TCDD developmental toxicity in zebrafish. Interestingly, a morpholino targeted against zfAHR1A does provide partial protection against PAH toxicity in zebrafish embryos (Incardona et al., 2005, 2006).

ZfARNT1 but Not ZfARNT2 Is Required for TCDD Developmental Toxicity in Zebrafish

To evaluate if the zfARNT2 protein is the functional dimerization partner for zfAHR2 *in vivo*, *zfarnt2* morphants and a line of insertional mutant zebrafish lacking expression of all forms of zfARNT2 (Golling et al., 2002; Prasch et al., 2004a) were assessed for responses to TCDD (Prasch et al., 2004b). Surprisingly,

it was discovered that zebrafish embryos with reduced or absent levels of zfARNT2 do not show protection against endpoints of TCDD developmental toxicity. In TCDD dose–response experiments, wild-type and *zfarnt2*$^{-/-}$ knockout embryos still exhibited similar hallmark toxicity responses to TCDD, including pericardial edema, reduced blood flow, and reduced lower jaw growth. The *zfarnt2*$^{-/-}$ embryos also exhibited zfCYP1A induction following TCDD treatment. This indicates that zfAHR2 signaling is functional in zebrafish embryos that lack zfARNT2. The sensitivity of *zfarnt2*$^{-/-}$ mutant embryos to TCDD suggested that an alternative form of zfARNT must be expressed that can form a functional heterodimer with zfAHR2. A search of the incomplete zebrafish genome revealed a likely zebrafish ortholog of the mammalian *arnt1*, and multiple splice variants of zfARNT1 were subsequently amplified and analyzed (Prasch et al., 2006). *In vitro* assays showed that zfARNT1 forms a functional heterodimer with zfAHR2 that binds AhRE sequences and promotes AhRE-driven transcription. The *in vivo* role of zfARNT1 in mediating TCDD developmental toxicity was then assessed using a morpholino designed against the translation start site of zfARNT1. The *zfarnt1*-MO was observed to provide partial to complete protection against endpoints of TCDD toxicity including pericardial edema, heart malformation, reduced cardiac output, ventricular standstill, reduced peripheral blood flow, reduced lower jaw growth, and zfCYP1A induction (Antkiewicz et al., 2006; Prasch et al., 2006). Thus, even though zfARNT2b can function with zfAHR2 *in vitro*, all *in vivo* findings support a mechanism by which TCDD toxicity is mediated by the zfAHR2 and zfARNT1 heterodimer.

Why Is Understanding AhR Signaling in Zebrafish Important?

Understanding AhR signaling in zebrafish is important because it provides a common mechanism for extrapolating TCDD toxicity results from the zebrafish model to other fish species, including lake trout. Recent findings on TCDD developmental toxicity in a marine fish species, red sea bream (*Pagrus major*), revealed that many endpoints of TCDD developmental toxicity were similar to those in zebrafish (Yamauchi et al., 2006). Furthermore, it appeared in TCDD-exposed red sea bream larvae that rsAHR2, like zfAHR2, mediated the induction of CYP1A and TCDD developmental toxicity (Yamauchi et al., 2006). This suggests that functionally characterizing the AhR signaling pathway in zebrafish might ultimately provide a foundation for both understanding and predicting differences between fish species in susceptibility to TCDD toxicity. In support of this idea, the discovery in zebrafish that TCDD developmental toxicity is mediated by zfAHR2 and zfARNT1 has caused scientists to focus on these transcription factors as the genes most likely to be affected in populations of killifish (*Fundulus heteroclitus*) that have evolved resistance to TCDD developmental toxicity. Similarly, functional understanding of the zebrafish AhR signaling pathway also may shed light on why vertebrate embryos generally are more sensitive to TCDD than adults. Finally, it is difficult to test for TCDD developmental and reproductive toxicity in a large number of freshwater, estuarine and marine fish species. On the other hand, it is feasible to determine if these species express AhR2 and ARNT1 and thereby have the capability of responding to TCDD; therefore, knowledge of which *ahr* and *arnt* genes mediate TCDD toxicity in fish is important in determining any effects TCDD and related AhR agonists may have on salmonine populations in the Great Lakes.

Induction of CYP1A Is Not Required for TCDD Developmental Toxicity in Zebrafish

A key question is whether or not induction of CYP1A is required for TCDD to cause early-life-stage toxicity in fish. The transcription factors zfAHR2 and zfARNT1 are required for TCDD to cause developmental toxicity in zebrafish; therefore, increased expression of one or more key genes mediated by TCDD activation of the AhR2/ARNT1 signaling pathway may lead to a subsequent cascade of molecular and cellular changes culminating in TCDD developmental toxicity in zebrafish. *Cyp1a* is the most well-characterized TCDD-regulated gene in fish. It contains AhRE sequences in its promoter and is directly regulated by the AhR/ARNT heterodimer (Abnet et al., 1999; Andreasen, 2002b; Powell et al., 2004; Whitlock, 1999); however, there has been considerable speculation about whether or not induction of *cyp1a* is required for TCDD developmental toxicity in fish.

Morpholino knock-down of *zfcyp1a* during embryonic development in zebrafish has provided an exciting opportunity to gain new insight about the potential role of CYP1A in mediating TCDD toxicity.

Although an initial study of *zfcyp1a* morphants indicated possible protection against TCDD-induced pericardial edema and reduced intersegmental blood flow at 72 hpf in zebrafish larvae (Teraoka et al., 2003), a subsequent study that examined, far more comprehensively, the developmental responses to TCDD at both 72 and 96 hpf in *zfcyp1a* morphants showed no protection whatsoever against several endpoints of TCDD developmental toxicity (Carney et al., 2004). More specifically, induction of zfCYP1A by TCDD was blocked to the same extent in zebrafish *zfahr2* morphants and zebrafish *zfcyp1a* morphants; however, in sharp contrast to the *zfahr2* morphants that were rescued from TCDD developmental toxicity, *zfcyp1a* morphants exposed to the same concentration of waterborne TCDD were not protected against any endpoint of TCDD developmental toxicity including the increase in pericardial edema, reduction in peripheral blood flow, inhibition of jaw cartilage growth, and impaired erythropoiesis. These latter findings are highly significant and mean that the key molecular events that lead to endpoints of TCDD developmental toxicity in zebrafish are not downstream of CYP1A induction; thus, the induction of zfCYP1A is merely a biomarker of TCDD exposure in zebrafish, and zfCYP1A is not involved in TCDD developmental toxicity (Figure 21.4). This realization has led aquatic toxicologists to search for other genes in zebrafish larvae whose expression is directly regulated by AhR2/ARNT1 heterodimeric binding to AhRE. Such genes would be required for certain endpoints of TCDD developmental toxicity. Three general approaches have been used: (1) the candidate gene approach, (2) microarray technology, and (3) computational approaches based on predicting functional AhRE sequences in promoter regions. These three approaches have revealed the complexity of the transcriptional response to TCDD, but no single, strong hypothesis has emerged about the identity of genes, other than *zfahr2* and *zfarnt1*, that lead to toxicity.

Recently research in zebrafish larvae that is focused on identifying genes that mediate specific endpoints of TCDD cardiac toxicity is beginning to change this picture (Carney et al., 2006b; Handley-Goldstone et al., 2005). AhR hyperactivation by TCDD during zebrafish larval development impairs heart growth, morphology, and function, culminating in mortality. Carney and coworkers (2006b) examined the transcriptional response to TCDD in the same adversely affected zebrafish larval hearts and compared it to the transcriptional response in the rest of the larval body. Zebrafish larvae were exposed to TCDD for 1 hour at 72 hpf. Hearts were extracted for microarray analysis at 1, 2, 4, and 12 hours after exposure (73, 74, 76, and 84 hpf). The remaining body tissue was also collected at each time for comparison. TCDD rapidly induced the expression of 42 genes within 1 to 2 hours of exposure. These genes function in xenobiotic metabolism, cell proliferation, heart muscle contractility, and pathways that regulate heart development. Importantly, these TCDD-induced changes in cardiac gene expression preceded the endpoints of cardiac toxicity, characterized by decreased stroke volume, reduced peripheral blood flow, and a halt in heart growth. The significance of this study is that it has identified candidates for AhR target genes in the fish heart of which we were previously unaware (Carney et al., 2006b).

Other exciting results are also now being obtained with molecular approaches in adult zebrafish exposed to TCDD; for example, TCDD inhibits caudal fin regeneration in the adult zebrafish, and microarray analysis of the regenerated tissue has revealed a profile of misexpressed genes suggesting an impairment of cellular differentiation and extracellular matrix composition that is potentially regulated by SOX9b (Andreasen et al., 2006). Taken together, the importance of this study and the previous studies on developmental cardiac toxicity (Carney et al., 2006b; Handley-Goldstone et al., 2005) are significant because they provide the first real glimpse into downstream cellular and molecular effects of zfAHR2 and zfARNT1 signaling that may ultimately lead to specific endpoints of TCDD toxicity (Figure 21.4).

Is TCDD Developmental Toxicity Dependent on AhRE Binding?

An alternative hypothesis for the mechanism of TCDD toxicity is that it requires nuclear localization of AhR but not AhRE binding followed by transcriptional activation of target genes. TCDD toxicity, according to this hypothesis, might be due to cross-talk between AhR signaling and the signaling of other nuclear proteins. This could occur either through direct interaction of the AhR with components of other pathways or through competition for shared transcription factors or cofactors. In either case, AhR activation may cause altered regulation of other signaling pathways resulting in toxicity; however, research findings obtained so far in zebrafish do not support a cross-talk mechanism of TCDD toxicity.

It is true that interactions between the zfAHR2 and hypoxia signaling pathways were observed in zebrafish (Prasch et al., 2004a), but there was no evidence to suggest this as a mechanism of TCDD toxicity. In addition, the *zfahr2* and *zfarnt1* morpholino studies demonstrated that both zfAHR2 and zfARNT1 are essential for the generation of developmental toxicity in zebrafish. The requirement for both proteins indicates that interactions of zfAHR2 with other signaling pathways or removal of zfARNT1 from other signaling pathways cannot be mechanisms mediating TCDD toxicity and suggests that the toxicity is more likely dependent on AhRE binding and alterations in gene expression caused by the zfAHR2 and zfARNT1 heterodimer. Nevertheless until specific genes and signaling pathways and their roles in mediating specific endpoints of TCDD developmental toxicity in zebrafish have been clearly identified, mechanisms of cross-talk with other signaling pathways cannot be ruled out.

Structure–Activity Relationships for PCB, PCDD, and PCDF Congeners as AhR Agonists

The AhR-mediated toxicity of PCBs, PCDDs, and PCDFs varies with the degrees of chlorination and patterns of chlorine substitution on the aromatic rings. There are 75 possible PCDD, 135 PCDF, and 209 PCB congeners, and the toxic potencies of these congeners vary by several orders of magnitude. The congeners are most potent when they have lateral chlorine substitutions that make them approximate isostereomers of 2,3,7,8-TCDD (Safe, 1990). PCDD and PCDF congeners with additional chlorination beyond the 2, 3, 7, and 8 positions or the loss of chlorine at either of these four positions decreases potency of the congener. The PCB congeners that are most potent have chlorine substitutions in the *meta* and *para* positions (positions 3, 4, or 5) that allow them to maintain a planar conformation. These non-*ortho*-substituted, planar PCB congeners include PCB 77, 81, 126, and 169. Chlorine substitutions in the *ortho* positions (positions 2 and 6) limit the planar conformation of the congener, and consequently mono-*ortho*-substituted PCB congeners are less potent in producing TCDD-like effects and di-*ortho*-substituted PCB congeners are inactive as AhR agonists. The mono-*ortho* substituted PCBs are far weaker AhR agonists in fish than in mammals and birds (Gooch et al., 1989). Mono-*ortho*-substituted PCBs are essentially inactive in causing early-life-stage mortality in rainbow trout (Walker and Peterson, 1991; Zabel et al., 1995a). This is probably due to reduced intrinsic efficacy of the mono-*ortho* PCBs as AhR agonists (Hestermann et al., 2000) in causing sac fry mortality rather than a pharmacokinetic difference, because mono-*ortho* PCBs are eliminated from rainbow trout larvae at essentially the same rate as planar PCBs which cause early-life-stage mortality (Zabel et al., 1995a).

TCDD Toxicity Equivalency Factor Approach

The TCDD toxicity equivalency factor (TEF) approach is used to characterize the risk of toxicity for organisms exposed to mixtures of PCBs, PCDDs, and PCDFs. TCDD is recognized as the most potent AhR agonist in vertebrates and therefore has been adapted as the prototype for estimation or calculation of relative toxicological potency (Safe, 1994). Congener-specific TEFs describe the potency of individual PCB, PCDD, and PCDF congeners relative to TCDD in causing AhR-related toxicity. The TEF approach assumes that congeners act by the same AhR-mediated mechanism and interact additively to produce toxicity. Because there are significant differences in the relative potency (REP) of certain AhR agonists, such as the mono-*ortho*-substituted PCBs which are essentially inactive in causing AhR-mediated early-life-stage toxicity in fish compared to birds and mammals (Walker and Peterson, 1991; Zabel et al., 1995a), it has been necessary for the World Health Organization (WHO) to derive fish-specific, bird-specific, and mammal-specific TEFs for individual PCB, PCDD, and PCDF congeners for use in ecological risk assessment (Table 21.1) (van den Berg et al., 1998).

Toxicity equivalency factors permit calculation of the AhR-related potency resulting from a mixture of PCB, PCDD, and PCDF congeners in an organism, or organ, based on chemical concentrations and the vertebrate class-specific potency values (TEFs). More specifically, REPs describe the potencies of individual PCB, PCDD, and PCDF congeners that are AhR agonists relative to TCDD. REPs are relative

TABLE 21.1

Toxic Equivalency Factors (TEFs) for Halogenated Aromatic Hydrocarbon Dioxin-Like Potency in Fish

Compound	TEF
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.5
1,2,3,6,7,8-HxCDD	0.01
1,2,3,7,8,9-HxCDD	0.01
1,2,3,4,6,7,8-HpCDD	0.001
OCDD	<0.0001
2,3,7,8-TCDF	0.05
1,2,3,7,8-PeCDF	0.05
2,3,4,7,8-PeCDF	0.5
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0001
3,4,4′,5-TCB (PCB-81)	0.0005
3,3′,4,4′-TCB (PCB-77)	0.0001
3,3′,4,4′,5-PeCB (PCB-126)	0.005
3,3′,4,4′,5,5′-HxCB (PCB-169)	0.00005

Note: World Health Organization ecological risk values can be found in van den Berg, M. et al., *Environ. Health Perspect.*, 106(12), 775–792, 1998.

potency factors of AhR agonists that are specific to an endpoint, life stage, and species, whereas TEFs are more generic potency estimates that are used for ecological risk assessments (van den Berg et al., 1998). A TEF is defined as the TCDD ED_{50} (effective dose in which 50% of test organisms show a response) or TCDD LD_{50} (effective dose in which 50% of test organisms are killed) divided by the ED_{50} or LD_{50} of the individual congener. For example, the fish-specific TEF for PCB 126 based on early-life-stage mortality in rainbow trout (Zabel et al., 1995a) is derived as follows:

$$\text{PCB 126 TEF} = \frac{374 \text{ pg/g wet egg (TCDD LD}_{50}}{74{,}000 \text{ pg/g wet egg (PCB 126 LD}_{50})} = 0.005$$

The concentration of a congener in an organism is multiplied by its respective TEF to determine the TEQ concentration contributed by that congener. TEQ concentrations for all the individual congeners in an organism are then added to calculate the total TEQ concentration contributed by the entire mixture of PCB, PCDD, and PCDF congeners present in the organism. To assess risk, this total TEQ concentration is compared to the administered dose or concentration of TCDD in the organism, determined in controlled laboratory studies, to cause toxicity.

Using lake trout as an example, early-life-stage mortality due to contamination of eggs with dioxin-like PCBs, PCDDs, and PCDFs can be evaluated by calculating the total concentration of TEQs in eggs (TEQ_{egg}). The TEQ_{egg} for a mixture of n congeners equals the sum of the TEQ_{egg} values for each congener (pg TEQ per g wet egg), which is the product of the concentration of the congener in the egg (C_{egg}; pg congener per g wet egg) multiplied by the fish-specific TEF of the congener (pg TEQ per pg congener), where i is a specific congener:

$$TEQ_{egg} = \sum (C_{egg})_i (TEF)_i$$

The egg concentrations of the 2,3,7,8-substituted PCDDs, PCDFs, and non-*ortho*-substituted PCBs are the AhR agonists that are routinely measured and included in this additive model of toxicity for fish (van den Berg et al., 1998).

Ecological Risk Assessment with Fish-Specific TEFs

Fish-specific TEFs for planar PCBs (i.e., PCB 77, 81, 126, and 169) and PCDDs and PCDFs that are AhR agonists in fish were determined for the endpoint of sac fry mortality in rainbow trout (Walker and Peterson, 1991; Zabel et al., 1995a). In addition, Zabel et al. (1995c) found that the TEF for PCB 126, based on sac fry mortality in lake trout (0.003), is similar to that for rainbow trout (0.005). These early-life-stage mortality-specific TEFs determined in rainbow trout following injection of graded doses of each TCDD-like PCB, PCDF, or PCDD congener into newly fertilized eggs (Walker and Peterson, 1991; Zabel et al., 1995a) were adopted by the World Health Organization's expert panel as fish-specific TEFs (van den Berg et al., 1998). To provide an additional degree of protection for fish early-life-stage toxicity, the actual TEF values determined for early-life-stage mortality in rainbow trout by Walker and Peterson (1991) and Zabel et al. (1995a) were rounded higher in half order of magnitude increments. Therefore, the fish-specific TEFs for the planar PCBs are typically more conservative than the original values of the specific studies and will generate slightly greater total TEQ values (Walker and Peterson, 1991; Zabel et al., 1995a).

The potency of AhR agonists has also been determined in fish cell cultures (Zabel et al., 1996). In fish, comparisons of potency values based on sac fry mortality, induction of ethoxyresorufin-*O*-deethylase (EROD) activity, and induction of cytochrome P4501A mRNA suggest that most congener potencies determined from cell culture endpoints are higher by an order of magnitude than the potencies determined for the same congeners based on sac fry mortality. In several individual congeners, however, the cell-culture-derived potencies are greater by more than one order of magnitude. In addition, it is generally observed that PCDD, PCDF, and PCB congeners are more potent in cell culture assays with regard to causing EROD induction and P4501A mRNA induction than in producing early-life-stage mortality (Cook et al., 1997).

The two major assumptions inherent in the TEF approach have both been validated for early-life-stage toxicity evaluation in salmonines. The first is that congeners interact additively to produce AhR-mediated toxicity; hence, toxicity may be predicted by summation of the TEQs calculated for each concentration of TCDD-like AhR agonist present in fish tissue. We designate these AhR agonists as being TCDD-like to indicate that they are the PCB, PCDD, and PCDF congeners shown in controlled laboratory studies to produce the same signs of early-life-stage toxicity in rainbow trout as TCDD. All of these congeners are full AhR agonists based on early-life-stage mortality, and the slopes of their dose–response curves in eliciting this response are similar (Walker and Peterson, 1991; Zabel et al., 1995a). The additivity assumption has been validated for predicting salmonine early-life-stage toxicity (Hornung et al., 1996; Zabel et al., 1995b); for example, when lake trout eggs were injected with a mixture of PCBs, PCDDs, and PCDFs mimicking that found in the eggs of feral Lake Michigan lake trout, the interaction was essentially additive (Walker et al., 1996). Additionally, graded doses of an organic extract made from Lake Michigan lake trout, injected into eggs of hatchery-reared rainbow trout (Wright and Tillitt, 1999) and lake trout (Tillitt and Wright, 1997), produced symptoms of dioxin-like toxicity (i.e., yolk-sac edema, craniofacial deformities, and hemorrhaging) in a dose-related fashion in both species. The TEF/TEQ approach for quantification of the doses suggests additive toxicity in both of these species. Non-additive effects of AhR agonists have been observed (Janz and Metcalfe, 1991), but those effects are more often observed for non-lethal endpoints.

The second major assumption of the TEF approach is that the toxicity of PCBs, PCDDs, and PCDFs is solely related to their TCDD-like toxicity. This assumption generally holds for predicting lethality in salmonine embryos and larvae (Cook et al., 1997; Zabel et al., 1995a). Validation of this notion has also been provided through the use of organic extracts of lake trout from the Great Lakes (Tillitt and Wright, 1997; Wright and Tillitt, 1999). Lake trout were collected and extracted with organic solvents to produce

a mixture of chemicals that directly reflected the mixture of hydrophobic chemicals present in Great Lakes lake trout at the time of collection. Subsequently, lake trout and rainbow trout eggs were exposed to graded doses of the extracts, and the dose-related mortality followed an additive model of toxicity (Tillitt and Wright, 1997; Wright and Tillitt, 1999). Other toxic effects of PCBs observed in mammals such as carcinogenesis, neurotoxicity, or endocrine disruption are, for certain mono-*ortho*- and di-*ortho*-substituted PCB congeners, not AhR-mediated (Safe, 1994); thus, non-AhR-mediated toxicity attributable to PCBs is not assessed by the TEF approach.

Early-Life-Stage Toxicity of PCBs in Trout

Laboratory toxicity studies with PCBs, PCDDs, and PCDFs (AhR agonists) have demonstrated the adverse effects of these compounds to early life stages of salmonines and support the interpretation that such toxicity is AhR mediated (Cook et al., 1997; Walker and Peterson, 1991, 1994a; Zabel et al., 1995a). Studies with PCBs have included early-life-stage toxicity characterizations based on the TEF approach. Zabel et al. (1995a) determined rainbow trout-specific REPs for PCBs that can be used in assessing risks to early-life-stage survival of lake trout. Of the 15 PCB congeners tested, only the non-*ortho*-substituted PCB congeners were toxic to early life stages of rainbow trout (in the order of potency of 126 > 81 > 77 > 169). *Ortho*-substituted PCB congeners, including 4, 28, 52, 105, 118, 126, 128, 138, 153, 156, and 170, were essentially inactive in causing signs of early-life-stage toxicity. This was true even at concentrations in the dosing solution that were approaching the solubility limit for these PCBs. Although early-life-stage toxicity in rainbow trout of the remaining 194 potential PCB congeners has yet to be tested, knowledge of the structure–activity relationship for such toxicity between the non-*ortho*-, mono-*ortho*, and di-*ortho*-substituted PCB congeners already tested suggests that the planar PCB congeners (PCB 77, 81, 126, and 169) will be the most significant contributors to TCDD-like developmental toxicity from exposure of lake trout eggs to complex mixtures of PCBs where non-AhR agonist PCB congeners are also present (Cook et al., 1997; Walker et al., 1996; Wright and Tillitt, 1999; Zabel et al., 1995a).

Some evidence suggests that certain PCB congeners that are not AhR agonists and are prevalent in lake trout eggs in the Great Lakes (such as PCB 153) are toxic to the early life stages of lake trout; however, the signs of early-life-stage toxicity are completely different from those produced by TCDD, and the egg concentration of PCB 153 required to elicit the response is greater than the egg concentration of this PCB congener in lake trout in the Great Lakes. More specifically, Broyles and Novek (1979) found that PCB 153, despite its lack of AhR agonist activity in fish (Vodicnik et al., 1981), caused early-life-stage mortality in Chinook salmon (*Oncorhynchus tshawytscha*) and lake trout at mean concentrations of 3,700,000 and 8,700,000 pg per g in the sac fry of each respective species. Importantly, the mortality observed by Broyles and Novek (1979) was not associated with blue sac disease, a sac fry toxicity syndrome that is the hallmark sign of toxicity of TCDD-like PCBs, PCDDs, and PCDFs (pers. commun., cited in Walker and Peterson, 1992). Hence, other PCB 153-related di-*ortho*-chlorinated congeners have the potential to produce early-life-stage toxicity in fish, albeit at egg concentrations much greater than those required for AhR agonists and possibly so high that they may not be environmentally relevant. PCB 126 is the most potent PCB AhR agonist in fish. As with TCDD, species differences exist with regard to its potency in causing early-life-stage mortality. Rainbow trout are less sensitive than lake trout, as evidenced by the LD_{50} for PCB 126 in rainbow trout (74,000 pg/g egg) being greater than in lake trout (29,000 pg/g egg) (Walker et al., 1991; Zabel, 1995a,c).

Additive, Synergistic, or Antagonistic Interactions of Congener Pairs

The major interaction between pairs of congeners to produce early-life-stage mortality in salmonines is additive interactions. Interactions between PCDDs, PCDFs, and PCBs and polybrominated dibenzo-*p*-dioxins (PBDDs), dibenzofurans (PBDFs), and biphenyls (PBBs) in producing rainbow trout and lake trout early-life-stage mortality have been investigated. As assessed by isobolographic analysis, the majority of congener pairs tested acted additively in causing mortality (Hornung et al., 1996; Zabel et al., 1995b); however, deviations from additivity (synergism or antagonism) have been detected in rainbow

trout embryos co-exposed to TCDD and PCB 77 or PCB 126 (Zabel et al., 1995b). Depending on the egg dose ratio of PCB 126 or PCB 77 to TCDD, cumulative mortality induced by the pair of congeners injected into the eggs is altered. PCB 126 is synergistic with TCDD at a dose ratio of 400:1; that is, the percentage of rainbow trout sac fry that died from co-exposure to PCB 126 and TCDD was greater than expected by summing the TEQs contributed by both compounds. PCB 77 was also found to be synergistic with TCDD at a dose ratio of 3500:1 and 4500:1 but antagonistic at a ratio of 1250:1. Bol et al. (1989) also found a synergistic relationship between PCB 77 and TCDD, or between PCB 77 and 2,3,4,7,8-PCDF, toward sac fry mortality in rainbow trout after waterborne exposure of rainbow trout sac fry. Synergistic activity between PCB 77 and TCDD was reported in rainbow trout for induction of hepatic AHH activity (Janz and Metcalfe, 1991) and for increased hepatic CYP1A protein levels and EROD activity in rainbow trout (Newsted et al., 1995). Thus, at certain egg concentrations there is a tendency for PCB 126 and TCDD and for PCB 77 and TCDD to interact synergistically in causing early-life-stage mortality in trout. In contrast, other congener pairs appear to interact additively or show no consistent pattern of synergism or antagonism. This difference, although slight, may have to be considered in ecological risk assessments for trout exposed to PCDDs, PCDFs, and PCBs where PCBs are the major contributor to the total TEQ concentration in eggs (Cook et al., 1997).

Toxicity of Mixture of PCBs, PCDDs, and PCDFs Toward Trout Early Life Stages

The complex interactions that might occur with mixtures of AhR agonist and non-AhR agonists have been investigated through a variety of techniques under controlled laboratory conditions. To understand the early-life-stage toxic effects of complex mixtures of PCDDs, PCDFs, and PCBs present in Lake Michigan salmonines, investigators have prepared mixtures of these compounds or extracted these compounds from feral fish and exposed rainbow trout or lake trout embryos to them. Additionally, Edsall (unpublished data, cited in Mac and Edsall, 1991) conducted a study with adult lake trout in which hatchery broodstock were fed a diet supplemented with a complex organic extract obtained from lake trout collected in 1984 from southeastern Lake Michigan. Egg hatchability and fry survival were significantly reduced in eggs from lake trout that had been fed and exposed in water to a high concentration of the extract. Unfortunately, no information on the total PCDDs, PCDFs, or planar PCB concentration in the lake trout eggs was provided, as this study predated routine analysis for these AhR agonists.

Direct injection of mixtures of HAHs into freshly fertilized trout eggs has also provided important information on the toxicity toward early life stages in salmonines. Wilson and Tillitt (1996) conducted a study in which a complex organic extract (containing TCDD-like PCBs, PCDDs, and PCDFs) was prepared from lake trout captured from Lake Michigan in 1988. In this study, fertilized rainbow trout eggs were injected with extracts containing 8.8 to 8800 ng/g total PCBs. The sublethal effects and mortality observed were consistent with TCDD-like toxicity. PCBs, PCDDs, and PCDFs contributed about one third each to the total TEQ concentration of the lake trout tissue extract (Wright and Tillitt, 1999). The same extract was also examined for its toxicity toward lake trout early life stages (Figure 21.5) (Tillitt and Wright, 1997). The toxicity of the extract was found to be additive in lake trout for lethality (Tillitt and Wright, 1997), as well as for sublethal endpoints of hemorrhage, edema, and craniofacial anomalies (Wright, 2006).

Lake trout and rainbow trout were exposed as eggs to either TCDD alone or a mixture of PCDD, PCDF, and PCB congeners at mass ratios mimicking those found in Lake Michigan lake trout eggs to study additivity (Walker et al., 1996). PCDD and PCDF congeners contributed approximately 45% to the total TEQ concentration of the dosing solution while the planar PCBs (77, 126, and 169) contributed 54%. Di-*ortho*-substituted PCBs (e.g., PCB 153) accounted for 98% of the congener mass of the dosing solution but were assumed not to contribute to the total TEQ concentration because PCB 153 does not produce signs of TCDD-like toxicity in trout (Walker and Peterson, 1991; Zabel et al., 1995a). Exposure of fertilized eggs to graded doses of the above simulated Lake Michigan lake trout egg PCDD, PCDF, and PCB mixture produced the same signs of early-life-stage toxicity, sac fry stage-specific mortality, and slope of the dose–response curve for mortality as TCDD (Walker et al., 1996). The LD_{50} values for the mixture—362 pg TEQ per g rainbow trout egg and 97 pg TEQ per g lake trout egg (calculated using

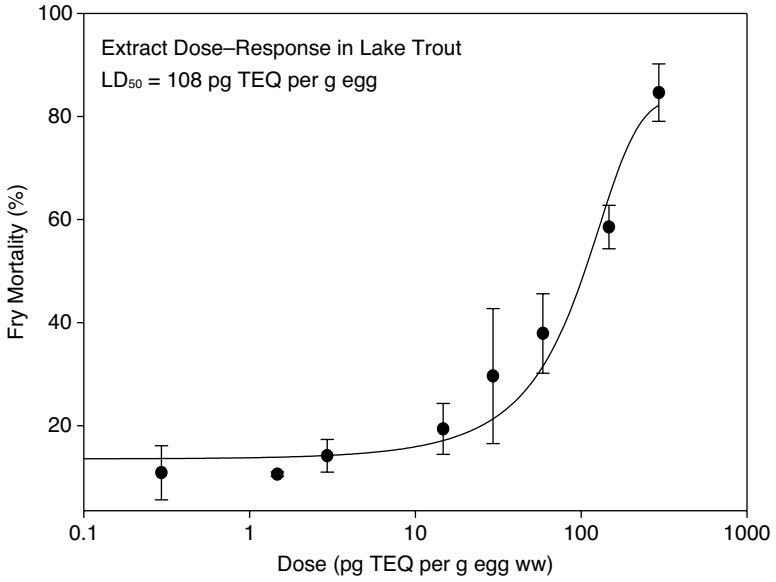


FIGURE 21.5 Embryo/fry mortality caused by graded doses of an extract of lake trout collected in Lake Michigan in 1988 injected into freshly fertilized, hatchery-derived lake trout eggs. Doses of the extract (TEQs) were based on the measured concentrations of HAHs and an additive model of toxicity. (Data are from Tillitt, D.E. and Wright, P.J., *Organohalogen Comp*., 34, 221–225, 1997.)

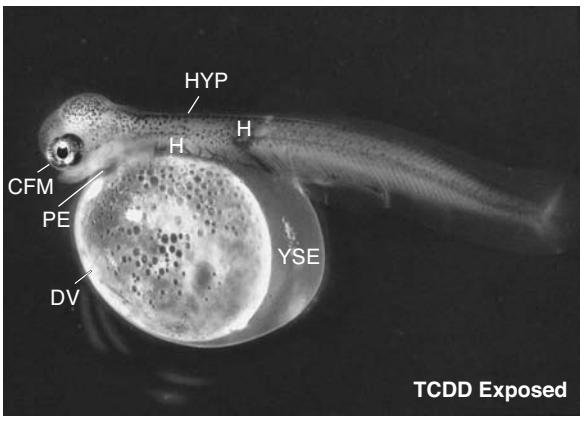
rainbow trout-specific REPs)—were 1.8- and 1.3-fold greater, respectively, than those for TCDD (200 pg TCDD per g rainbow trout egg and 74 pg TCDD per g lake trout egg). Although these results suggest a less than additive interaction for the TCDD-like PCB, PCDD, and PCDF congeners in the mixture, the TEQ LD_{50} and TCDD LD_{50} values for each species were only 30 to 80% different (Walker et al., 1996). Considering all of the potential variation in the model variables, this is a small amount of difference among the predicted and actual toxicity; consequently, additivity appears to be the appropriate model for HAHs in early life stages of salmonines. Furthermore, when the lake-trout-specific REP for PCB 126, 0.003 (Zabel 1995c), is used instead of the slightly more potent rainbow trout-specific REP for PCB 126, 0.005, to calculate the TEQ concentration of the mixture, then the TEQ LD_{50} and TCDD LD_{50} values for early-life-stage mortality in lake trout are no longer significantly different (Cook et al., 1997).

Symptoms of TCDD-Induced Early-Life-Stage Toxicity in Salmonines

Time Course

Lake trout embryos exposed to TCDD as fertilized eggs show stage-specific periods of sensitivity at hatch and later during the sac fry stage (Spitsbergen et al., 1991). The stage when toxicity occurs is determined by the egg TCDD concentration. At TCDD concentrations in eggs that are at or exceed the LD_{100}, toxicity is generally manifested at the time of hatching and is characterized by a high incidence of mortality. The affected lake trout embryos are typically incompletely hatched and have significant yolk sac edema. On the other hand, at lake trout egg concentrations of TCDD below the LD_{100} embryos hatch successfully and signs of toxicity are delayed until later during the sac fry stage. Mortality generally occurs from middle to end of the sac fry stage. Once the yolk sac has been absorbed and the lake trout fry begin feeding, TCDD begins to be more rapidly eliminated. Mortality during the fry stage is typically very low. Thus, the critical period for early-life-stage mortality in lake trout is from about one week prior to hatching until the end of the sac fry stage. There is typically no mortality in TCDD-exposed lake trout eggs before about one week prior to hatch. This does not include induction of CYP1A, which

A



B

FIGURE 21.6 Lake trout sac fry unexposed (top) and exposed (bottom) as fertilized eggs to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Sac fry exposed to TCDD have external signs of toxicity, including yolk sac edema (YSE) and pericardial edema (PE) associated with damage to vascular tissues (DV), hemorrhaging (H), craniofacial malformations (CFM), and hyperpigmentation (HYP), which lead to death prior to the swim-up stage of development. (From Cook, P.M. et al., *Environ. Sci. Technol.*, 37, 3867–3877, 2003. With permission.)

has been detected in lake trout embryos prior to hatch (Guiney et al., 1997, 2000). It is significant that all PCB, PCDD, and PCDF AhR agonists, tested as single compounds or as mixtures, produce identical signs of early-life-stage toxicity in trout that culminate in mortality from hatching until swim-up. At higher egg doses, signs of toxicity are invariably manifested earlier during the sac fry stage.

Pathologic Alterations

Spitsbergen et al. (1991) described the pathologic alterations in early life stages of lake trout exposed as newly fertilized eggs to an LD_{100} dose of TCDD (400 pg TCDD per g wet egg). Toxicity was first detected about one week prior to hatch and in larvae that survived hatching toxicity was manifested during the sac fry stage. The signs of TCDD toxicity in the lake trout sac fry (Figure 21.6) include the following:

- Retrobulbar, meningeal, subcutaneous, and pericardial petechial hemorrhages
- Severe subcutaneous edema of the yolk sac, with cessation of blood flow in the yolk sac and body

- Necrosis of the retina, brain, liver, and spinal cord
- Domed skulls and foreshortened maxillae
- Arrested development of skeletal and soft tissues

Most importantly, the above signs of TCDD toxicity are probably secondary to circulatory failure (Spitsbergen et al., 1991). Cardiovascular toxicity is first detected in TCDD-exposed lake trout sac fry as a reduction in perfusion of certain vascular beds and progresses gradually over a period of several days to complete cessation of blood flow. Accordingly, necrotic lesions in the retina, brain, liver, and spinal cord are probably secondary to the ischemia/anemia and hypoxia associated with this developmental cardiovascular toxicity (Spitsbergen et al., 1991). The insidious TCDD-induced circulatory failure is also considered responsible for the lethargy and arrested soft tissue and skeletal development in half-hatched embryos and sac fry and is the most probable cause of death. Decreased access to yolk sac nutrients, secondary to reduced perfusion of the vitelline vasculature, contributes to reduced growth of the sac fry and to mortality (Heming and Buddington, 1988). These findings of cardiovascular toxicity as a primary manifestation of TCDD toxicity in salmonines have been confirmed in subsequent mechanistic toxicological studies with zebrafish.

These gross and histopathologic signs of TCDD toxicity in lake trout sac fry are strikingly similar to blue sac disease in hatchery-reared salmonines (Spitsbergen et al., 1991). Both are expressed between hatching and swim-up culminating in mortality (Wolf, 1954); however, the etiology of blue sac disease is poorly understood. Physical or chemical stressors such as elevated ammonia, temperature shock, or hypoxia may trigger it (Ayles, 1974; Balon, 1980; Burkhalter and Kaya, 1977; Lasee, 1995; Wolf, 1969, as cited in Spitsbergen, 1991). Because the symptomology of blue sac disease (Wolf, 1954) is essentially identical to that produced by TCDD-like PCBs, PCDDs, and PCDFs in lake trout and other fish species the term *blue sac syndrome* has been coined to describe it (Walker and Peterson, 1994).

Spitsbergen et al. (1991) showed in lake trout embryos and larvae exposed to a LD_{100} dose of TCDD that mortality was greatest during the sac fry stage (~44%) followed by the hatching stage (~30%). Mortality of half-hatched TCDD-exposed embryos was apparently caused by their inability to effectively distribute hatching enzyme throughout the chorion which resulted in their dying only partially removed from the chorion (Spitsbergen et al., 1991). Some mortality was also detected during the egg stage (~9%), but it was substantially less than that observed during hatching or at the sac fry stage. Subsequent studies, conducted at lower egg concentrations of TCDD, have confirmed that mortality during the egg stage is exceedingly low compared to the sac fry stage (Walker et al., 1991).

Impaired Swim Bladder Inflation

Rainbow trout fry, exposed to TCDD as newly fertilized eggs that developed only mild yolk sac edema as sac fry, continually swam to remain off the bottom of the tank. This suggested an inability to maintain swim bladder inflation. A lack of swim bladder inflation is also a sign of TCDD toxicity in early life stages of Japanese medaka (*Oryzias latipes*) (Harris et al., 1994) and zebrafish (*Danio rerio*) (Henry et al., 1997). This was observed in lake trout embryos from Lake Michigan in the mid- to late-1970s, but has not been a consistent observation since that time in salmonine fry from Lake Michigan (Mac and Edsall 1991).

Impaired Cardiovascular Function

The cardiovascular system is a key site of action for TCDD (Cantrell et al., 1996, 1998; Guiney et al., 1997; Henry et al., 1997; Mizell et al., 1996; Spitsbergen et al., 1991). In TCDD-exposed lake trout, endothelial cells of yolk sac vessels, dorsal venule, dorsal arteriole, and sinusoidal endothelium of the liver displayed positive staining for CYP1A1 protein one week prior to hatch (Guiney et al., 1997). Endothelial cells showed the earliest positive staining for CYP1A1 of any tissue or cell type observed. A positive correlation between CYP1A induction in vascular endothelial cells and mortality of TCDD-exposed lake trout sac fry suggests that persistent stimulation of the AhR signaling pathway in the

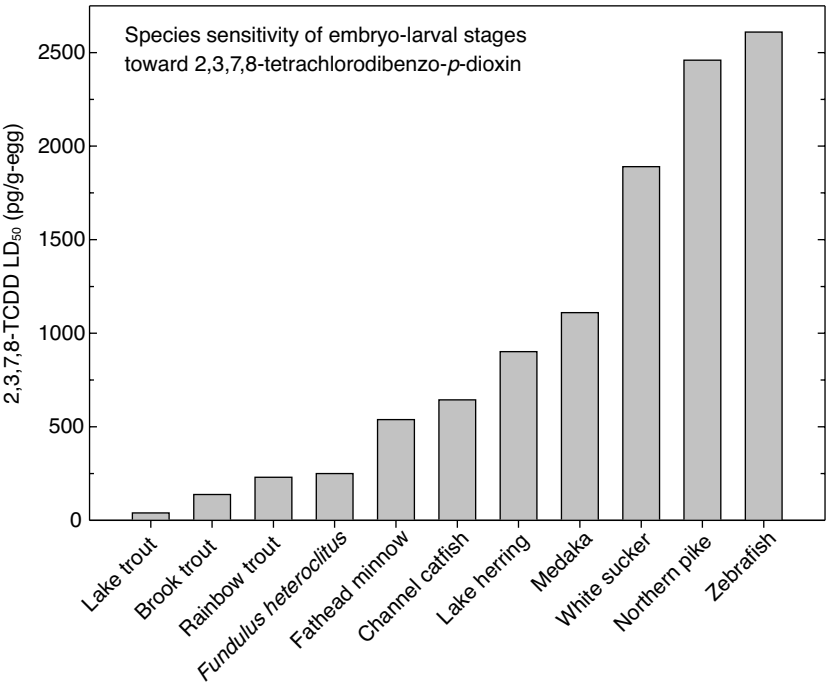


FIGURE 21.7 Species sensitivity towards 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Embryos were exposed as freshly fertilized eggs by waterbath during water hardening or egg injection prior to epiboli. (Data are from Elonen et al., 1998; Guiney et al., 1996, 1997; Helder, 1981; Henry et al., 1997; Toomey et al., 2001; Walker and Peterson, 1994b; Walker et al., 1991; Wright, 2006.)

vascular endothelium by TCDD may trigger edema and vascular dysfunction between hatching and swim-up; that is, a cascade of events initiated by AhR binding of TCDD may increase the permeability of vascular endothelial cells culminating in edema. In support of this interpretation, Guiney et al. (1998) showed that yolk sac edema fluid in TCDD-exposed lake trout is an ultrafiltrate of blood. What is not known is whether induction of CYP1A catalytic activity or some other event subsequent to AhR activation by TCDD in the vascular endothelium of lake trout sac fry is causally related to TCDD-induced cardiovascular dysfunction. Evidence from zebrafish indicates that induction of CYP1A in the endothelial cells or other locations of the developing fish embryo is not required for developmental effects of AhR-related toxicity (Carney et al., 2004); however, these same finding have not been demonstrated in trout, particularly lake trout. Although it is highly likely that the mechanistic findings related to the toxicity of AhR ligands observed in zebrafish are applicable to salmonine species, some verification of this model is needed.

Species Differences in TCDD Toxic Potency

The susceptibility of freshwater fish species to early-life-stage mortality caused by exposure of fertilized eggs to TCDD varies widely. The rank order sensitivity for 11 fish species shows that lake trout is the most sensitive, followed by brook trout and rainbow trout (Figure 21.7). The eight non-salmonine species are less sensitive. The rank order, beginning with the most sensitive fish species (based on egg TCDD LD_{50}), is lake trout (40 to 85 pg/g), brook trout (138 to 200 pg/g), rainbow trout (230 to 488 pg/g), Atlantic killifish (*Fundulus heteroclitus*) (250 pg/g), fathead minnow (539 pg/g), channel catfish (644 pg/g), lake herring (902 pg/g), medaka (1110 pg/g), white sucker (1890 pg/g), northern pike (2460 pg/g), and zebrafish (2610 pg/g) (Elonen et al., 1998; Guiney et al., 1996, 1997; Helder, 1981; Henry et al.,

1997; Toomey et al., 2001; Walker and Peterson, 1994b; Walker et al., 1991; Wright 2006). Brook trout and rainbow trout are about 3 to 6 times less sensitive than lake trout, whereas the other species are 8 to 38 times less sensitive. The only species that has demonstrated greater sensitivity than lake trout toward TCDD-induced toxicity is the bull trout (*Salvelinus confluentus*), also a member of the charr family (Cook et al., 2000). This latter study indicates that the bull trout has an LD_{50} value approximately one third that of the LD_{50} of lake trout for the early-life-stage toxicity of TCDD.

The reason for the greater risk posed by TCDD-like PCBs, PCDDs, and PCDFs to early-life-stage survival of lake trout is not known. Elonen et al. (1998) suggested that the ability of non-salmonine fish species to tolerate higher egg concentrations of TCDD might be related to their shorter development time to swim-up. The time from hatch to swim-up and first feeding ranged from 1 to 18 days for the non-salmonine species compared to 30 to 60 days for rainbow trout and up to 120 days for lake trout, the longest development time. Comparison of post-swim-up TCDD elimination rates between the non-salmonine species and lake trout suggests that lake trout with a long development time retain TCDD longer than species with short development times; however, this might not be the complete explanation. When induction of CYP1A mRNA is compared between rainbow trout and zebrafish cell cultures in response to graded concentrations of TCDD (a condition in which species differences in TCDD elimination rate is less likely to be a factor), TCDD is still less potent in eliciting an AhR-mediated response in zebrafish cells (Henry et al., 1997). This suggests that the species difference in potency of TCDD in causing AhR-mediated responses may involve species differences in the AhR signaling pathway (TCDD binding to AhR, dimerization of AhR with ARNT, DNA binding of TCDD/AhR/ARNT, or transactivation). Alternatively, the fish species that are most sensitive toward TCDD are also those species that are more oxygen sensitive. If, indeed, the cardiovascular system is an initial target for TCDD, species that are more sensitive to disruptions in oxygen homeostasis may be more sensitive to the untoward effects of dioxin.

Developmental Stages Sensitive to TCDD Toxicity

The lethal potency of TCDD is affected by the developmental stage at which exposure occurs (egg, sac fry, swim-up fry, or juvenile). In rainbow trout it has been clearly demonstrated that TCDD is most potent in causing early-life-stage mortality if administered immediately after egg fertilization. When TCDD is administered later in development, at the eye-up stage, at hatching, at the fry stage, or during juvenile development, it is progressively less potent in causing mortality. More specifically, the LD_{50} in rainbow trout exposed to TCDD as fertilized eggs (230 to 488 pg TCDD per g egg) is less than for TCDD exposure of swim-up fry (21 days post-hatch), where whole-body concentrations of 990 pg TCDD per g body result in 45% mortality (Mehrle et al., 1988; Walker et al., 1991). Even higher whole body concentrations (>5000 pg TCDD per g body) are required to produce mortality in juvenile rainbow trout (Spitsbergen et al., 1988). Thus, the developmental stage of trout, at the time of TCDD exposure, is an important factor in determining susceptibility to mortality with the sac fry stages being the most sensitive.

Route of TCDD Egg Exposure and Sensitivity to Toxicity

No significant difference was observed in the potency of TCDD to cause lake trout sac fry mortality when exposure of eggs to TCDD occurred via maternal transfer, waterborne exposure, or egg injection (Walker et al., 1994). The no-observable-adverse-effect level (NOAEL), lowest-observable-adverse-effect level (LOAEL), LD_{50}, and LD_{100} of TCDD were in the same range for all routes of TCDD exposure to lake trout eggs (Walker et al., 1994); thus, it was the egg dose of TCDD that determined toxicity, as opposed to the route of exposure. Dietary exposure of adult female brook trout and zebrafish to sublethal concentrations of TCDD has also been shown to cause toxicity in their embryos (Johnson et al., 1996; Wannemacher et al., 1992). In brook trout, the concentration of TCDD in eggs that resulted in dose-related increases in sac fry mortality was also similar following waterborne exposure and maternal transfer (Johnson et al., 1998; Walker and Peterson, 1994b).

Field Observations of Exposures and Effects in Great Lakes Salmonines

Correlations of Contaminants with Reproductive Success in Great Lakes Salmonines

Correlation analysis of chemical contaminant concentrations with observed effects in Great Lakes salmonines has been another method used to investigate the potential of a causal relationship among these variables. Although correlations are not absolute proof of causality, correlations can be a strong piece of evidence for causality in ecoepidemiology (Fox, 1991). Conversely, a lack of a correlation between a potential causal agent and the effect does not rule out a causal relationship but may simply imply greater complexity of any potential relationship. Correlation of organochlorine chemical concentrations with reproductive performance of salmonines in the Great Lakes has met with mixed success (Ankley et al., 1991; Fitzsimons, 1995; Mac et al., 1993; Zint et al., 1995). The experimental design for most of these studies consisted of collecting salmonine gametes from one or more of the Great Lakes, artificial spawning, rearing the eggs and fry in the laboratory, and observing stage-specific effects, including mortality. The observed effects were then correlated with chemicals measured in the adults, eggs, or rearing water. Correlations of PCB or HAH concentrations in the flesh of adults or eggs of Great Lake salmonines and embryo lethality have been significant in certain cases, while the correlations with fry mortality have been confounded and not significant in most cases.

The first work of this type on lake trout was conducted by Burdick et al. (1964) on fish collected from several lakes in upstate New York. Although not from the Great Lakes, their correlation of elevated contaminant concentrations (DDT) with reduced survival of fry proved to be important information on chemical effects in lake trout and as a model for future studies. They found no survival in lake trout fry that contained DDT over 2.95 μg/g wet weight (ww) (Burdick et al., 1964). Laboratory studies at the time with brook trout (Macek, 1968) confirmed the sensitivity of salmonines to DDT. Later studies found that adult lake trout fed diets with 6 μg DDT per g feed produced 100% mortality in their offspring (Burdick et al., 1972). Baltic salmon (*Salmo salar*) collected from the Baltic Sea indicated that PCBs could also have effects on egg survival and fry mortality (Jensen et al., 1970). These studies set the stage for the salmon- and trout-rearing studies with Great Lakes salmonines in which egg and fry survival was correlated to concentrations of organochlorine chemicals. One of the first rearing studies of lake trout eggs from the Great Lakes (Mac et al., 1985) found survival of eggs to hatching was greatest when the adults came from Lake Superior (96%) and lowest when the eggs were derived from females collected in Lake Michigan (70%). Poor survival was significantly correlated with the source of eggs, not the source of sperm and not the source of water in which the eggs were reared (Mac et al., 1985). Poor survival was observed in Chinook salmon swim-up fry derived from eggs that contained greater concentrations of dioxin-like chemicals (Ankley et al., 1991). They also found a correlation between hatching success and PCB content of eggs; however, survival of fry was not correlated to contaminant concentrations (Ankley et al., 1991). Later, Mac et al. (1993) found a significant negative correlation between total concentrations of PCBs and embryo survival to hatch in lake trout collected from Lake Michigan between 1977 and 1988. Fry mortality in those studies could not be attributed to disease or nutrition and was characterized by erratic swimming behaviors and loss of equilibrium prior to death (Mac et al., 1993). Other workers studying Chinook salmon found weak negative correlations between total concentrations of PCBs and survival (Edsall et al., 1993; Giesy et al., 1986) or no correlations at all (Fitzsimons, 1995; Smith et al., 1994; Williams and Giesy, 1992).

Simple correlations of chemical contaminants such as PCBs, TEQs, DDT, or mercury have not demonstrated completely consistent relationships with female-specific reproductive success. The reasons for inconsistencies are not evident; however, this fact likely speaks to the complexity of the stressors impinging on salmonine populations in the Great Lakes. Simple correlations of any type are highly unlikely to address all of the factors important for survival of salmonine offspring; yet, in a number of studies conducted over a number of years under a variety of conditions, elevated chemical contaminants have resulted in reduced survival in field-collected salmonines.

Field Observations of Dioxin-Like Pathologies in Great Lakes Salmonines

Dioxin-specific pathologies observed in salmonines in the Great Lakes provide further evidence for the adverse effects of HAH chemicals. Studies that measure survival and growth of Great Lakes lake trout or other salmonine species relative to concentrations of PCBs, HAHs, and resulting TEQs are critical to estimate effects of these chemicals on population dynamics; however, survival and growth can be generic endpoints if specific symptoms were not monitored in a study and, as such, may not provide the necessary linkages to specific chemicals required for establishment of causality. Chemical-specific responses have been found at the suborganismal level and help provide evidence for causal linkages between HAHs and organism-level effects in Great Lakes lake trout. In particular, chemical-specific pathologies associated with HAHs have been observed in both adult and early life stages of salmonines; thus, comparisons of observed pathologies in Great Lakes lake trout and other salmonines with dioxin-like symptoms of toxicity provide helpful diagnostic information for evaluation of cause-and-effect linkages (Fox, 1991).

A hallmark response of vertebrates to HAHs is induction of CYP1A1. Fish are no exception and exhibit a strong CYP1A1 response in a variety of tissues after exposure to HAHs (Stegeman and Hahn, 1994; Whyte et al., 2000). Evidence of a response in Great Lakes salmonines to HAH exposure first came from CYP1A1 induction in the tissues and gametes of lake trout collected in 1981 (Binder and Lech, 1984). Lake trout embryos spawned from adult lake trout collected from Lake Michigan or Green Bay had significantly induced aryl hydrocarbon hydroxylase (AHH) activity (CYP1A1 activity) relative to embryos from hatchery broodstock. Enzymatic characteristics of AHH induction by PCBs and inhibition by α-naphthoflavone (ANF) in hatchery trout offspring were identical to the enzymatic characteristics observed in offspring of Lake Michigan lake trout (Binder and Lech, 1984). Additionally, a reduction in AHH (CYP1A1) induction in offspring from the Lake Michigan lake trout was observed with increased age of the offspring, consistent with the reductions in PCB content of the offspring and indicating that dilution of the PCB concentration by growth of the offspring had occurred. Lake trout are known to be sensitive to CYP1A1 induction by the planar PCBs, such as PCB 126 (3,3′,4,4′,5-pentachlorbiphenyl), even at doses as low as 0.6 ng/g (Palace et al., 1996). Later in that same decade, Chinook salmon collected from Lake Michigan in 1987 were observed to have only slightly induced hepatic EROD activity (Ankley et al., 1989). At the same period in Lake Ontario (the late 1980s), lake trout were observed to have 6 to 62 times the hepatic AHH activity as lake trout collected from Lake Superior (Luxon et al., 1987). Lake trout collected from Lake Ontario in the beginning of the 1990s had only marginally induced EROD activity compared to lake trout collected at the same time from Lake Superior (Palace et al., 1998). No comprehensive evaluation of CYP1A induction has been conducted in Great Lakes fish, but the existing data are consistent with the concentration of HAHs measured in fish over the past three decades. The greatest amount of induction of monooxygenase activity was observed at the times when the HAH concentrations were highest, and subsequent investigations have observed decreasing hepatic CYP1A activity that corresponds with decreasing PCBs and other HAHs (see Figure 21.3) (DeVault et al., 1989; Hickey et al., 2006; Stow et al., 1995).

Hypothyroidism, thyroid hyperplasia, and altered thyroid status have also been observed in trout and salmon from the Great Lakes. Altered thyroid function is associated with HAH or dioxin-like toxicity in controlled laboratory exposures (Brown et al., 2004a; Kohn et al., 1996). Thyroid hyperplasia (Moccia et al., 1981) and altered thyroid hormones (Leatherland and Sonstegard, 1981) have been observed in several species of Great Lakes salmon from the mid-1970s through the 1990s (Rolland, 2000). Although other factors can cause thyroid hyperplasia and reductions in circulating thyroid hormones (iodine deficiency most notably), the existing experimental evidence for Great Lakes salmon suggests that the observed effects were not due to a general iodine deficiency (Leatherland and Sonstegard, 1984); however, the evidence that thyroid dysfunction observed in salmon and trout from the Great Lakes was linked to chemical contamination by HAHs is controvertible. Correlations of contaminant concentrations in Great Lakes salmon with the spatial extent of thyroid pathogenesis were never established (Leatherland, 1992, 1993; Moccia et al., 1977; Sonstegard and Leatherland, 1982). Additionally, laboratory treatments of fish with HAHs have not led to thyroid gland pathologies (Brown et al., 2004b; Grinwis et al., 2000; Leatherland and Sonstegard, 1978, 1979, 1980; Palace et al., 2001), even when thyroid hormone

concentrations in plasma have been depressed. Yet, when salmon from the Great Lakes were fed to rats, the rats developed thyroid hyperplasia, hypothyroidism, and goiters (Sonstegard and Leatherland, 1979). The same mechanism for thyroid toxicity in mammals is thought to occur in fish (Brown et al., 2004a; Kohn, 2000). Rats are known to be sensitive to PCB-induced thyroid dysfunction (Bastomsky, 1977; Bowers et al., 2004), so species sensitivity differences may account for the apparent lack of coherence in thyroid pathologies among fish and rodents. Conversely, fish-eating birds in the Great Lakes have had thyroid pathologies that corresponded with contaminant exposure (Rolland, 2000). We simply are not certain if HAHs had a bearing on the thyroid pathologies in Great Lakes lake trout and salmon that occurred in the 1970s to 1980s.

Histopathological lesions in the livers of afflicted lake trout from Lake Michigan were also consistent with an exposure and response to HAHs. Mac (1986) observed liver pathologies in fry from Lake Michigan collected during the late 1970s. These fry also had induced mixed function oxidases and depletion of liver glycogen, possibly indicative of the wasting syndrome that is a hallmark of HAH toxicity (Mac, 1986; Mac and Edsall, 1991).

Altered levels of vitamin A and its metabolites are another known effect of the dioxin-like toxicity of HAHs (Zile, 1992). Reduced or lowered amounts of vitamin A have been observed in fish collected from polluted waters of the Great Lakes (Rolland, 2000). White suckers (*Catostomus commersoni*) and lake sturgeon (*Acipenser fulvescens*) collected from the Ottawa River near Montreal also had reduced concentrations of vitamin A storage forms (retinyl palmitate and dehydroretinyl palmitate), and lake sturgeon had reduced concentrations of storage forms of vitamin A in the intestines (Branchaud et al., 1995; Ndayibagira et al., 1995). Unfortunately, no studies reported concentrations of retinoids in lake trout or other salmonines from the Great Lakes during the 20th century, so inferences from these other species are all we have to draw upon for field assessments of vitamin A levels. Laboratory studies with fish, mammals, and birds have all demonstrated that HAHs reduce plasma and storage levels of retinoids (Rolland, 2000; Simms and Ross, 2000). Specifically in salmonines, HAHs alter retinoid homeostasis in both adults (Gilbert et al., 1995; Ndayibagira et al., 1995; Palace and Brown 1994) and in developing embryos (Carvalho and Tillitt, 2004). Induction of monooxygenases in the liver, intestines, vasculature, and other organs by HAHs causes increased metabolism of retinoids (Branchaud et al., 1995; Simms and Ross, 2000; Zile, 1992). In conjunction with increases in phase I metabolism of retinoids, HAHs induce retinoid conjugation by glucuronyltransferases and enhance excretion of retinoids (Simms and Ross, 2000). These mechanisms for HAH-related reductions in retinoids are thought to be conserved across vertebrate species, including fish. Thus, vitamin A and its metabolites are known to respond to HAH exposure in fish, and reduced concentrations of vitamin A have been observed in Great Lakes fishes. Unfortunately, no direct measurements of retinoids in salmonine species from the Great Lakes exist for the period when HAH exposures were greatest.

Role of Other Stressors in Limitations of Great Lakes Lake Trout Recruitment

Chemical Contaminants Other Than HAHs

The other chemical contaminant that has been strongly considered and investigated as a potential factor in the lack of recruitment of lake trout in some of the Great Lakes is DDT. Generally, DDT fed in the diet has little effect on adult fishes. The threshold for toxic effects in brook trout eggs (*Salvelinus fontinalis*) is approximately 1.5 mg/kg ww, based on its effects in developing embryos and fry (Macek, 1968). There was generally little effect on the survival or hatching of eggs, but fry died at the swim-up stage. The threshold toxic concentration of DDT in lake trout eggs has been estimated to be approximately 5 mg/kg ww (Burdick et al., 1964). It should be noted that this was a field study and that it is unknown whether the concentrations of DDT present actually caused the adverse effects on egg viability and fry survival. The threshold to cause lethality of rainbow trout (*Oncorhynchus mykiss*) embryos is approximately 1.5 mg/kg, of egg (Hopkins et al., 1969). In studies of brook, brown (*Salmo trutta*), and lake trout, the thresholds for lethality of eggs and fry ranged from 5.8 to 11.9 mg/kg ww (Burdick et al.,

1972); thus, the concentration of DDT in salmonine eggs required to cause lethality was determined to be between approximately 1.0 and 10 mg/kg egg ww. The concentrations of DDT in salmonine eggs from the Great Lakes are currently in the range of 0.1 to 1 mg/kg egg ww (Hickey et al., 2006), depending on species and location. In 1984, the concentrations of DDT in Chinook salmon eggs from Lake Michigan were 1.2 mg/kg ww (Giesy et al., 1986). Historically, concentrations of DDT were as great as 20 mg/kg ww in eggs of fishes from the Great Lakes (Atchison, 1976; Burdick et al., 1964; Hopkins et al., 1969; Johnson and Pecor, 1969). When the correlation between rearing mortality was considered, some investigators found a correlation but others did not; for example, concentrations of DDT between 2.0 and 9.4 mg/kg egg ww in Coho salmon were not correlated with rearing mortality (Mason et al., 1967). In a similar study of the relationship between rearing mortality of Lake Michigan Chinook eggs and fry, no relationship was found between concentrations of the DDT complex and any of the hatching or survival of eggs or fry (Giesy et al., 1986); thus, the concentrations of DDT observed historically in Great Lakes salmonines were in the range of the threshold for adverse effects for over a decade and possibly longer (Figure 21.3). It is quite possible that DDT influenced lake trout fry survival in the Great Lakes.

Toxaphene is another persistent pollutant that has been found to be elevated in lake trout from the Great Lakes. Toxaphene was used primarily in the southern United States as an insecticide for cotton; however, atmospheric transport of this insecticide mixture and subsequent condensation in the temperate latitudes of the Great Lakes caused toxaphene to become elevated in lake trout of this region (Schmitt et al., 1990). The concentration of toxaphene in lake trout from the Great Lakes ranged from 8 mg/kg ww in the early 1980s to 0.1 to 2.0 mg/kg ww in the late 1980s (Gooch and Matsumura, 1985; Schmitt et al., 1990) to concentrations of less than 1 mg/kg by the mid-1990s (Hickey et al., 2006). Adverse effect thresholds of toxaphene toward reproduction and growth in fish have been estimated to be in the range of 0.4 to 0.6 mg/kg (Eisler and Jacknow, 1985; Mayer et al., 1975). No threshold has been established for adverse effects of toxaphene in lake trout. The concentrations of toxaphene in Great Lakes salmonines may have exceeded the predicted threshold for adverse effects on reproduction and growth during the 1980s and may well have been an additional stressor during this time.

Dietary exposure of fish to environmentally relevant concentrations of methylmercury can cause negative effects on neuroendocrine function and reproductive performance. The effect of mercury on steroid hormones and gonadal development was the focus of a recent study with tilapia (*Oreochromis niloticus*). Tilapia exposed to methylmercury chloride (slow-release capsule, interperitoneally) resulted in reduced steroid hormones and abnormal gonad development in females (Arnold, 2000). In another study, steroid hormones (both 17β-estradiol and 11-ketotestosterone) were suppressed in both female and male fathead minnows following a low dietary exposure to methylmercury (Drevnick and Sandheinrich, 2003). The study found retarded gonad development in females that corresponded to a reduction in 17β-estradiol and reduced spawning success. Thus, there is some evidence that methylmercury at environmentally relevant concentrations (<1 μg/g in diets) can reduce steroid hormones, alter gonad development, and impact spawning. The body burden of methylmercury that resulted in these impacts was in the range of 3 to 5 μg/g ww in the whole fish (Drevnick and Sandheinrich, 2003). Chronic effects on feeding behavior in grayling (*Thymallus thymallus*) were observed 3 years after a single acute exposure to the developing eggs (Fjeld et al., 1998). Concentrations of methylmercury in the grayling greater than 0.27 μg/g egg ww were expected to result in reduced feeding efficiency (Fjeld et al., 1998). Concentrations of methylmercury in Great Lakes lake trout were <0.3 μg/g whole carcass ww from 1976 to 1977 (May and McKinney, 1981) and <0.2 μg/g whole carcass ww in 1984 (Schmitt and Brumbaugh, 1990). Even though methylmercury is a reproductive toxicant with known effects on the hypothalamus–pituitary–gonad axis, the concentrations of methylmercury in Great Lakes salmonines over the last half of the 20th century did not exceed reproductive thresholds for adverse effects and would not have been expected to have contributed to the lack of recruitment observed in lake trout.

Polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in plastics, clothes, furniture, and numerous other consumer and industrial products over the past three decades (Hites, 2004). Production of PBDEs in the United States is currently 200,000 metric tons per year (Hale et al., 2003). The PBDEs used in plastics are not covalently linked to the plastic polymers and will leach out of the plastics as the products age; consequently, PBDEs are one of the few halogenated, persistent chemicals

that have been increasing in the aquatic environment of the Great Lakes during the last decade of the 20th century and first decade of the 21st century (Hites, 2004; Zhu and Hites, 2004). Concentrations of PBDEs in lake trout from the Great Lakes have been doubling every 3 years over the period from 1975 to 2005 (Zhu and Hites, 2004). The concentrations of total PBDEs are currently near 1000 ng/g lipid in lake trout collected from all of the five Great Lakes. The threshold concentrations for effects of PBDEs in lake trout, particularly with regard to reproductive function or developmental toxicity, are not known.

Loss of Breeding Habitat

The physical and chemical structure of the spawning habitat is another potential factor that could contribute to the loss of reproductive success in Great Lakes lake trout. Many factors need to be aligned for adult lake trout to return to the appropriate areas at the appropriate time and spawn. The spawning habitats must have proper substrate (size and shape), including interstitial spaces (depth); be at the correct water column depth; have suitable nursery areas nearby; and be of the correct size and water temperatures at spawning (Marsden et al., 1995). Lake trout spawn on reefs in the Great Lakes at depths from 5 to 80 meters (Goodyear et al., 1982). The spawning reefs have not been physically disturbed and continue to attract adult lake trout of spawning age in Lakes Michigan, Ontario, and Huron (Fitzsimons, 1995; Kelso et al., 1995; Marsden et al., 1995). Additionally, the hydrological conditions and temperature profiles that exist at these spawning areas were not thought to be a limitation for lake trout spawning at these areas in the Great Lakes for the last five decades of the 20th century (Marsden et al., 1995). Although pheromones and other olfactory cues were not understood well enough to evaluate (Marsden et al., 1995), the fact that large numbers of adult lake trout in spawning condition arrive annually at spawning reefs suggests that homing cues are not a limiting factor for their reproduction. The loss of spawning habitat, then, is not thought to constitute a significant factor in the rehabilitation of lake trout populations in the lower Great Lakes.

Predation

Parasitism of adult lake trout by the invasive sea lamprey was a key factor in the initial population crashes in the mid-20th century. The susceptibility of larval and juvenile age lake trout to parasitism has been considered an important factor in the inability to establish naturally reproducing lake trout populations in the Great Lakes. Since the population crashes of lake trout in the 1950s, the greatest amount of effort toward rehabilitation of lake trout in the Great Lakes has been focused on the control of sea lamprey populations (Selgeby, 1995). The success of the sea lamprey control program coupled with stocking programs resulted in large numbers of adult lake trout in the lower Great Lakes (Elrod et al., 1995; Holey et al., 1995); however, predation on early life stages of lake trout by forage fishes can cause significant mortalities and may be an important factor that is limiting lake trout populations today. In particular, alewife (*Alosa pseudoharengus*) prey on lake trout fry and are present on spawning reefs and nursery areas when fry emerge and swim up. As a result, they could limit recruitment of Great Lakes lake trout (Krueger et al., 1995); however, alewife populations have declined in Lake Michigan over the past four decades (Fleischer et al., 2000) and there are still no signs of recruitment in lake trout. Thus, the predation of early life stages of lake trout by forage fish, including alewife, may have been a contributing factor in lake trout recruitment failures, but probably not the most significant factor.

Thiamine Deficiency

A compelling argument can be made for the role of thiamine deficiency as a contributing or causal factor in the reproductive problems of lake trout in the Great Lakes from the 1980s to the present (Brown et al., 2005). Thiamine (vitamin B_1) is an essential nutrient required as a cofactor in glycolysis, the Krebs cycle, and the pentose pathway. Thiamine concentrations below approximately 1 nmol/g in eggs of lake trout result in early mortality syndrome (EMS) in the resultant fry (Fitzsimons, 1995). EMS is characterized by mortality of the fry at swim-up. Prior to death, the fry are hyperexcitable in response to external stimuli, have a loss of equilibrium, lay on their sides, swim in spiral patterns, are lethargic, and

generally show symptoms of neurological deficits (Marcquenski and Brown, 1997). The symptoms of EMS can be observed during incubation of lake trout eggs and fry collected from the Great Lakes (Brown et al., 2005). Other salmonines in the Great Lakes, such as Coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*) also exhibit varying degrees of thiamine deficiency and subsequent EMS in their offspring (Brown et al., 2005). In fact, some of the best data tracking the occurrence of EMS in Great Lakes salmonines can be found in Coho salmon reared as part of the restocking program of the Michigan Department of Natural Resources (Brown et al., 2005). Coho salmon fry, collected from adults spawning in the Platte River in Michigan, have been monitored since 1972 for mortality from hatch to feeding. The rates of fry mortality were <20% until 1979, when the rates of fry mortality increased and began to fluctuate but generally increased until this century (Brown et al., 2005). The EMS observed in Coho salmon from Lake Michigan during the late 1990s has been highly correlated with the thiamine content of the eggs (Wolgamood et al., 2005). This same extensive monitoring data for EMS is not available for other species or for the other Great Lakes; however, similar symptoms of EMS and low thiamine have been reported in salmonines from Lake Huron (Wolgamood et al., 2005) and Lake Ontario (Fitzsimons, 1995). The presumed cause of the thiamine deficiency in Great Lakes salmonines is a diet rich in thiaminase (Brown et al., 2005). Thiaminase is an enzyme that hydrolyzes thiamine and is present in large amount in alewife, a major food component of Great Lakes salmonines (Tillitt et al., 2005).

Case Study: Assessment of the Effects of AhR Agonists on Reproduction and Survival of Lake Trout in Lake Ontario

Application of toxicology data in actual ecological risk assessments can provide many insights. On the one hand, retrospective assessments can validate the applicability of data and models for assessment of both exposure and toxicity. Alternatively, the risk assessment process can facilitate refinements of the data and models, as well as revealing unanticipated uncertainties requiring further research. Examples of truly prospective toxicity risk assessments that are later validated are quite rare; however, the ultimate goal of ecotoxicological research is to develop a risk prediction capability that can be used to prevent damage to fish and wildlife populations in the future. Studies of the presence of persistent bioaccumulative toxicants such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) in aquatic food webs in sufficient concentrations to have impacted fish populations through AhR-mediated toxicities that affect the reproductive cycle can provide opportunities for validating toxicity risk prediction models and water quality criteria. A complete retrospective assessment must combine relevant toxicity and exposure data with integrated exposure, bioaccumulation, and chemical mixture toxicity models to predict population responses that are independently consistent with the species' population histories for the ecosystem.

The most complete evaluation of the potential for application of AhR-mediated toxicity data and models to fish is provided by a retrospective study of the extirpation of lake trout (*Salvelinus namaycush*) in Lake Ontario which occurred around 1960 (Cook et al., 2003). Toxicological models of AhR agonists have been rigorously developed and tested for salmonines and even specifically for lake trout. The sensitivity of lake trout has been established (Table 21.2), and the additivity of HAHs to cause dioxin-like toxicity in early life stages of salmonines has been confirmed through a number of different approaches. The relative potency factors (REPs) used to derive the WHO toxicity equivalency factors (TEFs) for the AhR agonists were almost solely based on rainbow trout early-life-stage mortality studies associated with concentrations in embryos, so direct application to lake trout embryos should provide good estimates of potency of the congeners. Moreover, there have been numerous signs of dioxin-like toxicity in both adult and early life stages of lake trout from the Great Lakes, particularly Lakes Michigan and Ontario. Cook et al. (2003) chose to relate lake trout early-life-stage mortality to dose measured as the 2,3,7,8-TCDD toxicity equivalence concentration in lake trout eggs (TEQ_{egg}) for known AhR agonists:

$$TEQ_{egg} = \sum (C_{egg})_i (TEF)_i$$

TABLE 21.2

Mortality Thresholds and LD_{50} Values for 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD) in Early Life Stages of Lake Trout

NOAEL (pg/g)	LOAEL (pg/g)	LD_{50} (pg/g)	Exposure	Ref.
—	40	40–80	I	Walker et al. (1991)
—	—	47	I	Guiney et al. (1997)
30	—	44	EI	Guiney et al. (1996)
34	—	53	EI	Guiney et al. (1996)
23	50	58	MT	Walker et al. (1994)
41	—	65	EI	Guiney et al. (1996)
34	40	69	EI	Walker et al. (1994)
45	—	69	EI	Guiney et al. (1996)
—	—	74	I	Walker et al. (1996)
44	55	80	I	Walker et al. (1994)
20	40	81	I	Wright (2006)
—	—	85	EI	Zabel et al. (1995c)

Note: Exposure routes were by egg injection (I) or egg immersion (EI) during water hardening or by maternal transfer (MT).

The risk evaluation component missing from historic analyses of the effects of HAHs on lake trout populations has been an accurate model for the exposure assessment. Analytical methods for HAHs were improving during the last two decades of the 20th century, but accurate, reliable methods for quantification of HAHs were not consistently applied to this problem until the 1990s. Separation technologies for analytical clean-up, most notably carbon-based enrichment of the planar PCBs, PCDDs, and PCDFs, were developed in the 1980s (Kuehl et al., 1984; Smith, 1981) and only became routinely used in the Great Lakes in the 1990s (Echols et al., 1995; Feltz et al., 1995; O'Keefe et al., 1990). So, the ability to accurately measure HAHs in lake trout only became common after the concentrations of HAHs had severely dropped in the Great Lakes. These improved analytical methods have been applied to archived samples of lake trout to examine historic trends in contaminant concentrations, but archived samples of lake trout collected prior to 1970 are generally not available; therefore, an accurate exposure assessment of HAHs in lake trout in the first half of the 20th century has been the limiting factor in our ability to critically evaluate HAH impacts on lake trout populations and recruitment. The retrospective risk analysis of HAH on lake trout from Lake Ontario conducted by Cook and colleagues (2003) offers the most complete exposure assessment available combined with examination of the consistency between the predicted lake trout early-life-stage mortalities and actual lake trout population changes during the 20th century.

Lake Trout Exposure History Assessment for PCBs, PCDDs, and PCDFs in Lake Ontario

The retrospective analysis of HAH impacts on lake trout in Lake Ontario conducted by Cook et al. (2003) used the ecological risk assessment model of the USEPA (1998) and the recommendations for application of TEFs to fish and wildlife. The tissue and life stage targeted for the exposure model were lake trout eggs, as the developing embryos are known to be the most sensitive life stage, and maternal deposition of the HAHs into developing oocytes is the major route of exposure to the eggs (Walker et al., 1991; Zabel et al., 1995). The concentrations of HAHs in lake trout eggs had to be estimated for many of the years in question, because egg samples were unavailable for analysis prior to 1978. The authors used sediment concentrations of HAH congeners from core samples, along with biota-sediment accumulation factors (BSAFs), to estimate concentrations in lake trout eggs (Ankley et al., 1992). Lipid-normalized egg concentrations (C_{egg}) were estimated from organic carbon-normalized sediment concentrations (C_{soc}) of HAHs by the formula:

$$C_{egg} = (C_{soc})(BSAF_{egg})$$

The C_{soc} values were measured from 1-cm section samples of two radionuclide-dated sediment cores. Two radionuclides (^{210}Pb and ^{137}Cs) were used to estimate the chronologies of the sediment cores (Goldberg, 1963; Krishnaswami et al., 1971; Robbins and Edgington, 1972), along with two dating methods, to verify the accuracy of the aging (Appleby and Oldfield, 1978; Robbins, 1978; Robbins and Herchle, 1993). The aging estimates derived from the two methods were nearly identical, as were the resolutions provided by each estimation technique (Cook et al., 2003). $BSAF_{egg}$ values were measured from samples collected from 1978 to 1988. These empirical values for $BSAF_{egg}$ intrinsically account for differences in bioaccumulation for each of the congeners, as these factors are specific to species, life stage, tissues, and locations. For periods prior to 1972 the $BSAF_{egg}$ values required adjustment due to the fact that larger concentrations of the chemicals existed in the water relative to concentrations in the surface sediments. Because BSAFs are by definition normalized to C_{soc}, increased uptake of the chemicals via water (pelagic food chain) with respect to uptake via sediment (benthic food chain) will increase the $BSAF_{egg}$ values in proportion to the amount of increased bioaccumulation by lake trout (Burkhard et al., 2003).

The expected pre-1970s greater $BSAF_{egg}$ values were modeled based on predicted decreases in the sediment–water column concentration quotients ($\prod_{socw}$) (Cook and Burkhard, 1998; Thomann et al., 1992):

$$\prod_{socw} = \left(C_{soc}\right) / \left(C_w^{fd}\right)$$

The values of $\prod_{socw}$ for Lake Ontario were modeled with dynamic mass balance simulations (Endicott and Cook, 1994). Based on predicted changes in $\prod_{socw}$, $BASF_{egg}$ values prior to 1970 were predicted to be two to three times greater than more recent values; thus, the pre-1970 $BASF_{egg}$ values were set at two times the measured values (1978 to 1988) by Cook et al. (2003). The BASF values ranged from >10 to 0.3 for the PCBs and 0.27 to <0.001 for PCDDs and PCDFs and were consistent with other published data and predictions (Russell et al., 1999). The concentrations of all of the dioxin-like HAH congeners measured in the aged sediment core slices were used to estimate concentrations of each congener expected to be present in lake trout eggs over the period from approximately 1920 to 1988. The modeled concentrations of PCBs, PCDDs, and PCDFs in lake trout eggs were verified by comparison with measured values in lake trout collected in 1988, 1990, and 1991. The modeled concentrations were in good agreement with the measured amounts. For the periods when no lake trout were present in Lake Ontario, BSAF-derived lake trout egg concentration estimates were also verified based on modeling from concentration of HAHs measured in herring gull eggs (Environment Canada, 1991). Again, estimates from both of these methods were in good agreement with one another (Cook et al., 2003).

2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (2,3,7,8-TCDD) Toxicity Equivalence Risk Modeling in Lake Trout from Lake Ontario

Lake trout egg concentrations of HAHs predicted from the sediment core profiles were then multiplied by congener-specific TEF values (Table 21.2) and summed according to an additive model of toxicity (van den Berg et al., 1998). The resulting TEQ_{egg} values estimated in lake trout eggs over the period from 1920 to 2000 were then compared to toxicity thresholds of TCDD for early-life-stage mortality in laboratory studies (see Table 21.1). Cook and his colleagues (2003) estimated that lake trout egg TEQ_{egg} values less than 30 pg/g ww would not result in embryo or sac fry mortality, while concentrations greater than 100 pg/g ww would result in 100% mortality, and that mortality would be approximately linear between the concentrations of 30 and 100 pg/g (Figure 21.8). There was a general trend of increasing TEQ_{egg} values in lake trout eggs starting from 1930 and increasing to a maximum predicted amount in the late 1960s (Figure 21.8). Following the 1960s, TEQ_{egg} values steadily decreased. The 2,3,7,8-TCDD toxicity equivalence risks posed by AhR agonists over time suggest that mortality in lake trout fry would have been first expected as early as 1940 and continued to be expressed until the early 1980s, a 40-year period. Complete mortality of fry would have occurred for approximately three decades from 1945 until about 1975 (Figure 21.8) based on the estimated TEQ_{egg} values; thus, regardless of any other stressor

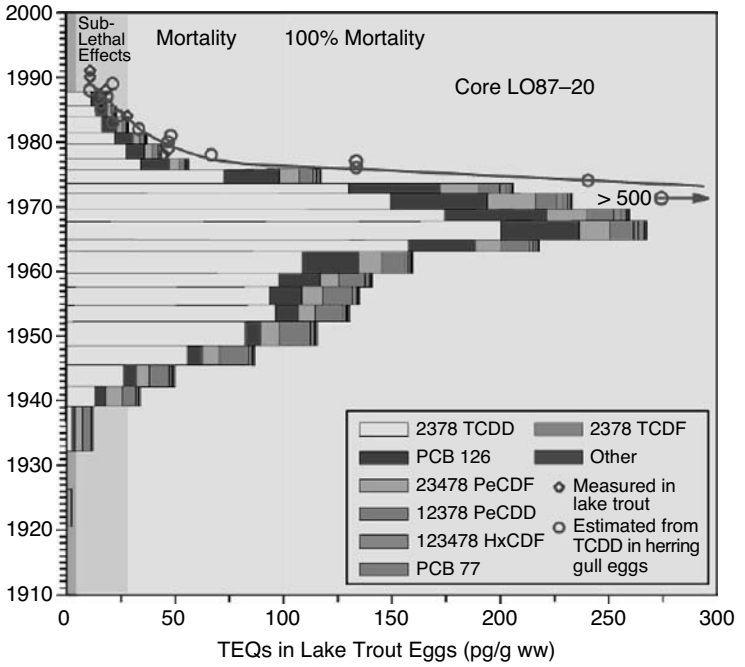


FIGURE 21.8 TEQs in lake trout eggs determined retrospectively from analysis of radionuclide-dated, 1-cm sections of a sediment core (LO87-20) from eastern Lake Ontario. The concentrations of each AhR agonist times the appropriate BSAF values for lake trout eggs and fish TEF equate to the contribution of each chemical to the TEQs in the eggs that may be related to mortality expected from acute and chronic toxicity in Lake Ontario lake trout sac fry. Predicted TEQs in eggs are compared to measured TEQs in eggs of lake trout (diamonds) and lake trout egg TEQs estimated from TCDD concentrations measured in herring gull eggs (circles). (From Cook, P.M. et al., *Environ. Sci. Technol.*, 37, 3867–3877, 2003. With permission.)

present, TCDD-associated toxicity would have impacted the lake trout population in Lake Ontario for at least four decades (1940 to 1980) and would have caused complete reproductive failure for nearly three decades (1945 to 1975). These predictions could underestimate impacts to the extent some AhR agonists were present and unaccounted for in the assessment.

The resulting estimates of early-life-stage mortality predicted over this time period were then compared to known rates of fry mortality and observation of population trends in Lake Ontario (Figure 21.9). The minimum toxicity model had thresholds as listed above. The maximum toxicity model incorporated the precept that sublethal toxic effects, which occur during development, would result in losses from the population at later life stages. Sublethal effects of HAHs in salmonine fry most certainly could affect juvenile fitness and their ability to compete in natural environments; therefore, predictions of maximum toxicity presented in their risk assessment were based on a TCDD threshold for mortality of 5 pg/g and 100% mortality occurring at 50 pg/g in the lake trout eggs. Their choice of a maximum toxicity threshold of 5 pg/g was also based on the fact that lake trout from Lake Superior had similar exposures during the 1960 and 1970s, yet there were no associated reductions in native trout populations (Hansen et al., 1995).

The predicted lake trout early-life-stage toxicities were compared with the observed sac fry mortality rates in lake trout collected by the New York Department of Environmental Conservation (Cook et al., 2003). The observations of blue-sac-related mortality compared well with the predicted estimates based on the additive 2,3,7,8-TCDD toxicity equivalence model over the period from 1978 to 1991 (Figure 21.9). Additive toxicity of HAHs had previously been demonstrated with binary mixtures, synthetic mixtures, and complex environmental mixtures, so the 2,3,7,8-TCDD toxicity equivalence approach was supported and again appeared to accurately reflect the toxicity of HAH congeners in an environmental mixture.

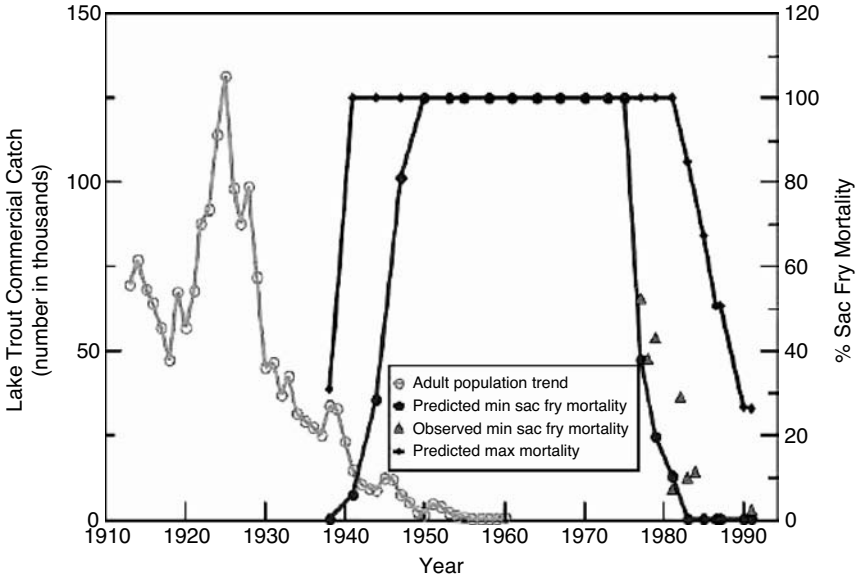


FIGURE 21.9 Integrated sediment core analysis and toxicity model predicted lake trout sac fry mortality (acute and chronic toxicity related) in comparison to lake trout population decline prior to extirpation around 1960 and blue sac syndrome mortality observed for sac fry raised from fertilized eggs collected from stocked fish between 1976 and 1990. (From Cook, P.M. et al., *Environ. Sci. Technol.*, 37, 3867–3877, 2003. With permission.)

There was an observed concordance between population fluctuations in the numbers of adult lake trout and stocking rates of a unique lake trout fry stocking program (Figure 21.10). A lake trout fry stocking program (Elrod et al., 1995) could account for increases in lake trout prior to 1925 and the dramatic decline in lake trout numbers recorded after 1925 through the 1940s (Figure 21.10). Most interesting from an epidemiological perspective was the appearance of several strong year classes of adult lake trout that appear to be associated with earlier peaks in numbers of stocked fry. The last peak of these strong year classes of lake trout was observed around 1939 and indicates that lake trout reproductive success occurred as late as 1934. The epidemiological evidence for natural reproduction was consistent with the predicted maximum toxicity risk analysis (Figure 21.9) presented by Cook et al. (2003). Finally, the first signs of natural reproduction observed in 1986 (Marsden et al., 1988) are also consistent with the maximum AhR-mediated toxicity risk model for reproductive success of lake trout in Lake Ontario (Figure 21.9). A remaining uncertainty is whether AhR-mediated effects on lake trout fecundity may have further impacted reproductive success during the long HAH exposure history in Lake Ontario.

This retrospective risk assessment provides strong evidence along multiple lines of evidence that HAHs had impacts on populations of lake trout in the Great Lakes in the latter half of the 20th century. Predictions of toxicity agree with observations of sac fry mortality from the late-1970s to mid-1980s (Figure 21.9), are consistent with lake trout population estimates for Lake Ontario (Figure 21.9), and match up well with exposure metrics estimated from other monitoring programs (Figure 21.8).

Summary

The history of lake trout populations in the Great Lakes during the 20th century is certainly a complex story of human disturbance, exotic invasive species, and changes in water quality. The dynamic interaction of these stressors on populations of lake trout has been difficult to assess and has lent itself to a variety of interpretations. The evidence for effects of overfishing and sea lamprey predation on lake trout population crashes experienced in the mid-century has been assumed to be conclusive by fisheries scientists; however, we have summarized information regarding the plausibility of chlorinated hydrocarbons, in

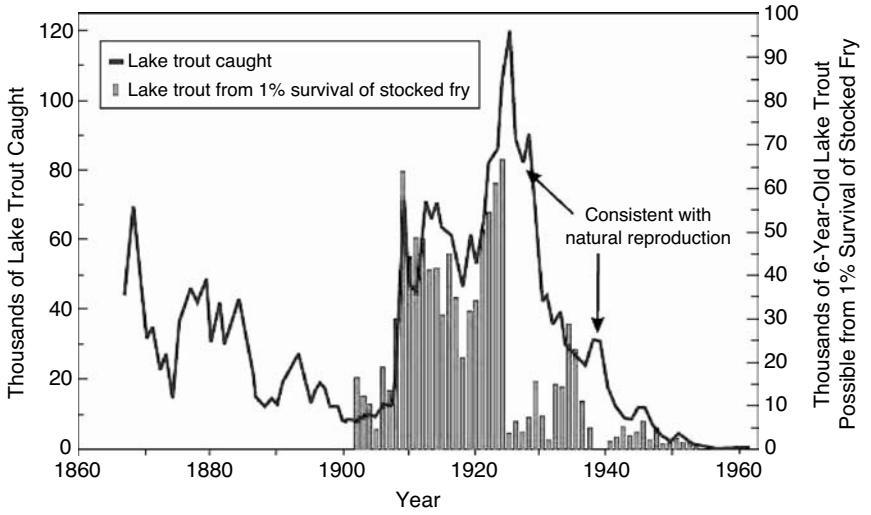


FIGURE 21.10 Association of lake trout population levels, as revealed by annual commercial catch (thousands) prior to 1940, with numbers of 6-year-old lake trout (thousands) predicted to be available for catch based on hypothetical survival of 1% of fry annually stocked into Lake Ontario. The commercial catch population data show evidence for recruitment up until about 1936 of additional lake trout from adults that grew from fry stocked earlier. (From Cook, P.M. et al., *Environ. Sci. Technol.*, 37, 3867–3877, 2003. With permission.)

particular HAHs, having had an influence in the crash and subsequent lack of rehabilitation of lake trout populations in the lower Great Lakes for three to four decades during the 20th century. In this section, we evaluate the information covered in this chapter from the perspective of an ecoepidemiological assessment. The criteria for establishment of cause-and-effect linkages in ecotoxicology have been taken from the biomedical sciences and adapted for use with fish and wildlife species (Fox, 1991). The ecoepidemiological criteria put forth by Fox (1991) provide a framework for evaluation of the strength of evidence for causation of an effect (i.e., lack of recruitment in Great Lakes lake trout populations) with a putative causal agent (i.e., HAH chemicals). Seven criteria provide the basis for evaluation of the data or information for causation in this approach. The ecoepidemiological approach was used by Mac and Edsall (1991) to evaluate the strength of data that HAHs had an effect on lake trout populations in the Great Lakes. Those authors concluded that the evidence at the time supported the hypothesis that chemical contaminants had caused reproductive impairment in lake trout populations of the Great Lakes; however, they went on to state that much of the evidence was circumstantial, with little definitive proof of such a causal relationship. Since the time of that evaluation, a great deal of additional evidence has served to strengthen and clarify the causal relationship between HAHs and reproductive impairment of lake trout in the Great Lakes. A large portion of that evidence has been presented in this chapter and is summarized here within the ecoepidemiological framework.

Ecoepidemiological Criteria

The seven criteria for evaluation of the relationship between a suspected causal agent and an adverse effect are (1) probability, (2) time-order, (3) strength of association, (4) specificity, (5) consistency of association, (6) predictive performance, and (7) coherence. We provide a summary of the existing data within this context. The hypothesis that we are evaluating with regard to these criteria is that HAHs were of sufficient concentrations in lake trout populations to cause embryo and fry mortality and subsequent lack of recruitment in the lower Great Lakes from the 1950s until the early 1980s. In our evaluation, we will compare and contrast evidence from all of the Great Lakes except Lake Erie. This is because Lake Erie never had very strong lake trout fisheries or populations due to the relatively shallow nature of the lake and the hydrological patterns of the lake.

Probability

What is the probability of HAHs causing such an effect in lake trout of the Great Lakes? This criterion is viewed not necessarily in the statistical sense but in the biological sense of probability. Could it be probable that HAHs elicit such an effect in lake trout? In this sense, effects of HAHs on lake trout populations were most certainly a possibility and indeed a probability. Lake trout were exposed to elevated concentrations of HAHs from the initial production and release of these chemicals into the Great Lakes ecosystem until after they were banned in the late 1970s (Figure 21.7 and Figure 21.8). Lake trout are among the most sensitive fish species tested with HAHs (Elonen et al., 1998; Spitsbergen et al., 1991; Walker et al., 1991), with embryo and fry mortality occurring at doses as low as 40 pg TCDD per g egg (Spitsbergen et al., 1991; Walker et al., 1991, 1994) and sublethal effects of hemorrhage and yolk sac edema occurring at doses as low as 2 to 15 pg TEQ per g egg with environmental mixtures of HAHs (Tillitt and Wright, 1997; Wright, 2006). Both measured (DeVault et al., 1986, 1989) and estimated (Cook et al., 2003) concentrations of TEQs in the eggs of Great Lakes lake trout exceeded these thresholds for toxicity during the three-decade period in question. Thus, strong direct evidence and plausible logic indicate a reasonable probability for HAHs/TEQs to have had adverse effects on lake trout reproduction and development during this period; therefore, the criterion of probability appears to be met.

Time-Order

Did the production and release of HAHs into the Great Lakes environment precede the adverse impacts observed on lake trout reproduction? Production of PCBs was first reported in Anniston, Alabama, and the first large-scale production of PCBs in the United States was by the Monsanto Corporation at their Sauget, Illinois, facility in 1929 (Durham and Oliver, 1983). PCBs were used initially by industry as dielectric fluids in capacitors and electrical transformers, then their popularity increased and their use spread to other applications. The annual usage rate of the most popular PCBs (Arochlor® 1242) increased in the United States to 18 million pounds/year in 1957 and over 50 million pounds/year by 1970 (Cairns et al., 1986). The presence of PCBs, PCDDs, and PCDFs in the Great Lakes has been documented in the sediment record since the 1930s (Cook et al., 2003; Durham and Oliver, 1983). The most precise and presumably accurate account of HAH concentrations and exposure in Great Lakes sediments during the 20th century was described for Lake Ontario (Cook et al., 2003). Concentration estimates of HAHs and TEQs in lake trout from approximately 1930 to 1990 indicated that toxicity thresholds of TEQs in lake trout were exceeded as early as 1940. By 1950, in Lake Ontario the concentrations of TEQs in the eggs of lake trout were great enough to cause 100% mortality in the resultant embryos or fry (Cook et al., 2003). This timeframe coincides with the extinction of the lake trout from the lower Great Lakes, the early 1950s in Lake Ontario (Figure 21.9) and Michigan (Figure 21.2).

Of course, heavy commercial fishing pressure depleted stocks of adult lake trout in all of the Great Lakes, including Lake Superior, but only in the lower Great Lakes of Ontario, Michigan, and Huron were lake trout extirpated. Lake trout in Lake Superior always maintained self-sustaining populations throughout this period. The predation of adult lake trout by sea lamprey in Lake Superior was less due to smaller populations of the lamprey in Lake Superior (Smith and Tibbles, 1980). Lower rates of sea lamprey predation in Lake Superior may have been an additional factor in the survival of those populations; yet, only in the lower Great Lakes, where the concentrations of chemical contaminants exceeded thresholds for toxicity to lake trout, did the populations of lake trout fail completely. Even stocking of hundreds of thousands of lake trout into the lower Great Lakes during the 1970s was not sufficient to bring about a self-sustaining population. So, based on the temporal coincidence of rises in dioxin-like contaminant concentration and increases in reproductive failure of lake trout in the lower Great Lakes, the time-order criterion is supported.

The criterion of time-order is also supported by the biological data on monitoring of hatching and fry survival of lake trout from Lake Michigan and Lake Ontario. Mac and Edsall (1991) summarized the temporal trends of hatching and fry survival over the period from 1975 to 1988. Lake trout eggs collected from adult fish from southeastern Lake Michigan had a clear increasing trend in survival from 1975 to 1988, roughly 65% to 100% over this period (Mac and Edsall, 1991). Fry survival over this same period also had a similar increase, with the exception of 1975 and 1977; however, protocols in the early years

of this monitoring called for the removal of "deformed fry" from the cultures (Willford et al., 1981), which may have confounded the fry survival data. The temporal aspects of lake trout hatch and fry survival studies from Lake Ontario also support this criterion. The New York Department of Environmental Conservation monitored lake trout sac fry mortality associated with blue sac syndrome from 1977 to 1984 (Cook et al., 2003). The decrease in blue-sac-related fry mortality during this period was consistent with the decreases in TEQs and predicted toxicity over this period (Figure 21.8) (Cook et al., 2003). Thus, the reports of signs of blue sac symptoms in lake trout sac fry and subsequent mortality rates from the mid-1970s to the late 1980s followed a temporal trend consistent with general declines of HAHs (Lake Michigan) and predicted toxicity, based on TEQs in lake trout (Lake Ontario). In each case, the time-order criterion is supported. Data are not available for either contaminant concentrations or blue sac syndrome in lake trout from Lake Huron.

Strength of Association

This criterion refers to the strength to which there is an association between the putative causal agent and the effect across wide geographic areas. This criterion also refers to the magnitude in differences in effects observed in exposed vs. non-exposed populations. Was there a coincidence between HAHs in lake trout populations across the Great Lakes and symptoms of blue sac syndrome or reproductive failure? The evidence for this comes from comparisons of concentrations of dioxin-like contaminants in lake trout over the past 50 years. Clearly, lake trout and other fish from the lower Great Lakes (Michigan, Ontario, and Huron) had greater concentrations of HAHs as compared to lake trout from Lake Superior (Baumann and Whittle, 1988; DeVault et al., 1986, 1989; Hickey et al., 2006). This difference in chemical contamination of the Great Lakes continues even today, with Lake Superior having smaller concentrations of PCBs, as well as a number of other persistent organic pollutants, as compared to Lakes Michigan, Ontario, and to a lesser extent Lake Huron (Hickey et al., 2006). There was a consistent pattern of the incidence of blue sac syndrome in lake trout during the late 1970s into the 1980s when fry rearing studies were conducted (Cook et al., 2003; Mac et al., 1985). Little or no blue-sac-related mortality was observed in lake trout fry from Lake Superior, while elevated amounts of this syndrome were observed in fry from Lake Michigan (Mac et al., 1985). This is consistent with the elevated HAH chemicals observed at the time in lake trout from Lake Michigan and HAHs at concentrations below known thresholds of toxicity in lake trout from Lake Superior. The most complete and compelling evidence for the strong association among lake trout exposure to dioxin-like chemicals (HAHs), predicted toxicity, and degree of observed blue-sac-related mortality comes from the retrospective risk analysis for Lake Ontario (Cook et al., 2003). In that example, we saw that estimated exposure of lake trout to dioxin-like PCBs, PCDDs, and PCDFs was high over a four-decade period, from roughly 1945 to 1985. Predicted toxicity of TEQs derived from the PCBs, PCDDs, and PCDFs in lake trout eggs (TEQ_{egg}) was sufficient to cause complete lake trout sac fry mortality and lack of recruitment in lake trout inhabiting Lake Ontario over this period. Consistent with that premise, no recruitment was observed over that period in lake trout populations from Lake Ontario.

The geographic distribution of dioxin-like pathologies observed in lake trout and other salmonines of the Great Lakes supports the strength of association between HAH exposure and HAH-induced toxicity in lake trout. The most consistent of the dioxin-like responses observed in both lake trout adult and sac fry from contaminated areas was induction of CYP1A activity. AhR-mediated induction of CYP1A is a biomarker of exposure to TCDD-like AhR agonists and can be measured catalytically as AHH or EROD activity (Stegeman and Hahn, 1994). Induction of CYP1A was observed in lake trout sac fry from Lake Michigan during the late 1970s (Binder and Lech, 1984). EROD was also induced in Chinook salmon from Lake Michigan in the mid-1980s (Ankley et al., 1989), and, most significantly, a direct comparison demonstrated up to 60-fold greater AHH induction in lake trout from Lake Ontario compared to those collected in Lake Superior (Luxon et al., 1987). A histological finding that is consistent with dioxin-like toxicity and has been observed in Great Lakes salmonines was thyroid hyperplasia (Moccia et al., 1981) and altered thyroid hormones (Leatherland and Sonstegard, 1981); however, a geographic distribution of thyroid pathologies in salmonines from the Great Lakes was not found to be linked to chemical contamination (Leatherland, 1992, 1993; Moccia et al., 1977). Although a thyroid abnormality known to be associated with HAH-induced toxicity was present in Great Lakes salmonines

during the time of their greatest exposures to HAHs, a correlation between HAH exposure and thyroid dysfunction was never established.

Specificity

The criterion of specificity requires investigators to evaluate how specific the biological outcomes are in relation to the disease-causing agent. In other words, can the observed symptoms of the disease be explained by other factors or causal agents? More specifically, could blue sac syndrome and stage-specific mortality observed in Great Lakes lake trout be caused by factors other than HAHs? Further, could the lack of recruitment observed in populations of lake trout from Lakes Michigan, Huron, and Ontario over the period of greatest HAH exposure have been specific to the one factor of elevated concentrations of HAHs? The specificity of dioxin-like symptoms of toxicity in lake trout sac fry following exposure to TCDD-like AhR agonists is fairly unique among chemical-induced toxicities. From the perspective of contaminant-induced effects, the specificity criterion is supported by the blue sac syndrome observed in lake trout; consequently, this supports the hypothesis that HAH-induced sac fry mortality occurred in Great Lakes lake trout. The specific signs of yolk sac edema, hemorrhage in the vitelline vasculature and trunk, craniofacial anomalies, lack of swim-bladder inflation, circulatory failure, and stage-specific mortality between hatch and swim-up are all well known in dioxin-treated salmonines (Helder, 1981; Spitsbergen et al., 1988, 1991; Tillitt and Wright, 1997; Walker et al., 1991; Wright, 2006). This suite of developmental toxicity endpoints is also conserved across various families of fish (Elonen et al., 1998; Walker and Peterson, 1994a). The receptor-mediated mechanisms of HAHs in developing fish embryos that have been elucidated in the zebrafish are consistent with what is known regarding molecular mechanisms of dioxin-like toxicity in salmonines. These mechanisms of toxicity are conserved across taxa, as well as within a taxonomic class such as teleost fishes. Thus, the presence of blue sac disease in Great Lakes lake trout from the mid-1970s to 1980s was consistent with AhR-related toxicity. Some of the endpoints of toxicity observed in lake trout swim-up fry from Lake Michigan (Mac and Edsall, 1991) or Lake Ontario (Burdick et al., 1964, 1972) during this period resembled those associated with DDT (Macek 1968). However, the embryo-larval signs of DDT toxicity in salmonines are not similar to AhR-induced blue sac syndrome. It is reasonable to hypothesize that even though the major symptom of toxicity observed in lake trout during this period were associated with dioxin-like toxicity of PCBs, PCDDs, and PCDFs, simultaneous DDT contamination of eggs may have influenced survival and subsequently recruitment.

The name *blue sac syndrome* was first coined to identify a condition observed in hatchery-reared salmonines (Wolf, 1954). Under hatchery conditions, blue sac syndrome could be triggered by a variety of different stressors including ammonia, temperature shock, or hypoxia (Balon, 1980; Burkhalter and Kaya, 1977; Lasee, 1995; Spitsbergen et al., 1991; Wolf, 1969). The suite of symptoms described in the literature for these hatchery fish resembled AhR-induced toxicity so much that Walker and Peterson (1994a) adopted the same term, blue sac syndrome, to describe dioxin-related toxicity. The specificity criterion for establishing cause-and-effect linkages between HAH exposure and stage-specific mortality in Great Lakes lake trout is not supported by these water quality factors; however, no information available indicates that ammonia, temperature, or dissolved oxygen was outside of acceptable levels in the spawning areas for lake trout in the lower Great Lakes (Elrod et al., 1995; Holey et al., 1995). Thus, the specificity criterion is supported in the fact that there is little likelihood that factors other than HAHs could have caused the pathologies, blue sac syndrome, and stage-specific mortality observed in Great Lakes lake trout.

Consistency of Association

This criterion refers to the ability to observe the same cause-and-effect relationship over time, across geographic regions or locations, in different species at the same locations, and by different researchers using a variety of approaches. It is sometimes referred to as *consistency of replication* (Fox, 1991), but it is hoped that replication of contamination events on this scale will not reoccur. So, in ecoepidemiology consistency of association must generally refer to the consistency of the observed relationship over space, time, and species. More specifically, for this case, we must evaluate the consistency of the relationship between HAH contamination in the aquatic food webs of the Great Lakes and either a lack of recruitment or symptoms of dioxin-like toxicity in the exposed fish.

The temporal association of either the lack of recruitment or the symptoms of dioxin-like toxicity with HAH contamination in Great Lakes salmonines has been consistent and supports the hypothesis. The best example of temporal consistency of the association has come from the retrospective risk analysis of HAH exposure in lake trout from Lake Ontario (Cook et al., 2003). The declines in lake trout populations during the 1930s in Lake Ontario were temporally consistent with elevated HAH exposure (Figure 21.9). Additionally, declines in sac fry mortality during the late 1970s and early 1980s were temporally associated with a decline in HAH exposure of lake trout (Figure 21.9). Further evidence of a consistent association between HAH exposure and dioxin-like toxicity observations in Great Lakes lake trout over time comes from the observations of fry and sac fry mortalities in lake trout from Lake Michigan (Mac and Edsall, 1991). These authors reported consistent improvements in hatchability and sac fry mortality in lake trout from Lake Michigan over the period of 1975 to 1984. This was at a time when the concentrations of PCBs were declining in the aquatic ecosystem of Lake Michigan in general and lake trout from Lake Michigan in particular (Figure 21.3).

The geographic distribution of symptoms of dioxin-like toxicity in lake trout was consistent with the relative degree of contamination in the Great Lakes. Dioxin-like effects were observed in early life stages of lake trout in all three of the lower Great Lakes with historic populations, while problems of reduced hatchability, or elevated sac fry mortality were not observed in progeny of Lake Superior lake trout (Mac and Edsall, 1991). Thus, consistency of association is supported based on the geographic distribution of elevated contaminant burdens, particularly PCBs and dioxins (DeVault et al., 1989; Hickey et al., 2006) and the observations of dioxin-like endpoints of toxicity in developing fry from those lakes (Cook et al., 2003; Mac and Edsall, 1991; Mac et al., 1985, 1993).

Consistency of association is also supported by the fact that the same signs of toxicity were observed in different species at the same locations. Skea and coauthors (1985) observed sac fry mortality consistent with dioxin-like toxicity in rainbow trout, Chinook salmon, and lake trout from Lake Ontario. Researchers from New York observed similar effects in lake trout and rainbow trout collected in this same time period (Cook et al., 2003). Similarly, the Michigan Department of Natural Resources reported that Chinook salmon fry from Lake Michigan were observed to have similar stage-specific mortality from 1979 to 1981 (Mac and Edsall, 1991), consistent with the observations in lake trout at the same time (Mac et al., 1985, 1993). The fact that multiple species from the same locations expressed elevated fry mortality supports the consistency of association criterion.

Predictive Performance

The criterion of predictive performance requires that a deductive hypothesis drawn from the association can predict an unknown fact or consequence (Fox, 1991). In two cases, the association of HAH contamination in Great Lakes lake trout and the lack of recruitment observed during the last half of the 20th century led to predictions that were confirmative of this relationship. One of the predictive models that strongly supported this association was the retrospective analysis of Lake Ontario outlined above (Cook et al., 2003). In that case, sediment core HAH profiles were predictive of the population levels of lake trout, the signs of sac fry mortality, and reductions in fry survival. This is a strong affirmation of the association, and provided a predictive model for the outcomes observed in lake trout from Lake Ontario.

This criterion was also satisfied through the fulfillment of Koch's postulates. Heinrich Herman Robert Koch, the noted German bacteriologist and epidemiologist, published a series of four postulates that, when satisfied, provide evidence for causality between an agent and a disease. These postulates have been adopted by environmental toxicologists to help identify causal linkages between chemical contaminants and adverse effects in exposed organisms (Giesy et al., 1994). Adapted for toxicology, Koch's postulates state that: (1) an agent (chemical) must be present in the affected organisms, (2) the agent must be correlated with the observed symptoms of the disease, (3) the agent must be isolated and identified (measured) from the affected organism, and (4) the agent must be introduced into healthy individuals and cause the untoward symptoms of the disease. These postulates have been satisfied through the isolation and extraction of chemicals from lake trout from Lake Michigan and the reintroduction of these extracts into healthy, freshly fertilized trout eggs (Tillitt and Wright, 1997; Wright and Tillitt, 1999). The extract of lake trout from Lake Michigan produced graded, dose-dependent symptoms of blue sac syndrome in lake trout embryos from the hatchery (Tillitt and Wright, 1997) and rainbow trout embryos (Wright and

Tillitt, 1999). The extracts were able to induce dioxin-like symptoms of toxicity that were predicted by the concentrations of PCBs, PCDDs, and PCDFs measured in the extract. The dose–response relationships developed in these experiments produced LD_{50} values based on the TEF/TEQ approach that matched previously measured values in lake trout and rainbow trout. The complex mixture of HAHs taken from lake trout from Lake Michigan was able to induce the same endpoints of toxicity observed in the feral fry, and this relationship was predicted by the concentrations of HAHs and an additive model of toxicity. This was strongly affirmative evidence which meets the criterion of predictive performance.

Coherence

The coherence criterion refers to the coherence of information on the association with known facts about life history, biology, and toxicology. Is the information about the association consistent with this knowledge? Is the relationship biologically plausible? Does the information conflict in some way with theoretical models? Can the suspected agent cause the symptoms and effect? This criterion is generally evaluated from the basis of coherence with theoretical models, biological knowledge, and toxicological information about the association. We have seen through the detailed studies in zebrafish the mechanisms whereby dioxin and dioxin-like chemicals (e.g., HAHs) produce their characteristic signs of blue sac syndrome in developing embryos and larvae. Although the mechanism of dioxin toxicity in lake trout has not been elucidated to this same extent as it has in zebrafish, the endpoints of HAH toxicity are the same in both these species and a similar pathway through the AhR occurs in salmonines, including lake trout. The sensitivity of lake trout toward dioxin has been established (Spitsbergen et al., 1991; Walker et al., 1991) and indeed is the most sensitive species of fish tested to this point (Elonen et al., 1998). Complementary to the sensitivity of lake trout toward dioxin and other HAHs, the exposure of lake trout to HAHs has also been established in the lower Great Lakes (Baumann and Whittle, 1988; DeVault et al., 1989; Hickey et al., 2006). Certainly, confounding exposures to other organochlorines, most notably DDT and its metabolites, also occurred during this time (Baumann and Whittle, 1988; Hickey et al., 2006). DDT can cause a swim-up syndrome in salmonines (Macek, 1968) and may well have contributed to the lack of recruitment observed in lake trout from the lower Great Lakes; however, concentrations of DDT in lake trout were not found to be above toxicity thresholds on a consistent basis. Based on the exposures of lake trout to HAHs during the last half of the 20th century and the sensitivity of this species toward HAHs, it is certainly plausible that an association between HAHs in the Great Lakes and the lack of recruitment in lake trout occurred over that time period.

Biological field data is also coherent with the premise of an association of HAHs and the lack of recruitment of lake trout in the lower Great Lakes during this same period. Pathologies of dioxin-like toxicity were observed in adult lake trout during this time, as well as in the developing fry. Fry from the lower Great Lakes developed blue sac syndrome and suffered from swim-up mortalities that were consistent with HAH-induced effects. During this same period, lake trout from Lake Superior had significantly smaller concentrations of HAHs, were not observed to have symptoms of HAH toxicity, and were not suffering from a lack of recruitment into the population. Thus, the biological information on lake trout from the Great Lakes supported the plausibility of this association.

Finally, the toxicological information, particularly our knowledge regarding dose–response relationships for HAHs in lake trout, supports a causal relationship between HAHs and population-level effects in lake trout from the Great Lakes over the last half of the 20th century. Correlative studies comparing concentrations of chemicals in salmonine eggs taken from the Great Lakes with mortality have reported conflicting results. A number of studies found no relationship between HAH contaminants in eggs and early-life-stage mortality of the resulting fry (Fitzsimons, 1995; Williams and Giesy, 1992; Zint et al., 1995), while others have observed a correlation between these factors (Ankley et al., 1991; Mac and Schwartz, 1992; Mac et al., 1993). This type of experimental design, however, is fraught with problems and might not allow clear relationships to be resolved. Many factors that may well contribute to mortality in the developing fry may not be measured; for example, the quality of the accuracy of chemical measurement of HAHs has changed and improved over the past two decades, so early studies may not have quantified the HAHs or other chemicals accurately. Additionally, most of the correlative studies did not measure all of the HAHs known to be present, and many of these studies were conducted at a time when the concentrations of HAHs in salmonines from the Great Lakes were reduced and near

predicted thresholds, making correlations difficult at best. So, some of these studies do not fully support a relationship between lake trout egg exposure to HAHs and lake trout recruitment failures.

Field studies that have demonstrated a dose–response relationship between HAHs and lake trout effects offer the strongest support for the coherency criterion and an association between these factors. Graded doses of organic extracts from Lake Michigan lake trout were injected into hatchery-derived eggs and caused blue sac syndrome and mortality in a dose-related fashion (Tillitt and Wright, 1997, Wright and Tillitt, 1999). Not only did these studies demonstrate the toxicity of the chemicals present in lake trout from the Great Lakes, but they were also consistent with the additive models of toxicity that had been developed for HAHs in salmonines (Zabel et al., 1995b,c). As such, they support the coherence criterion as being consistent with known toxicological models. The retrospective risk assessment of HAHs in Lake Ontario (Cook et al., 2003) indicated that estimated exposure from HAHs was sufficient to cause complete mortality in lake trout sac fry from Lake Ontario from approximately 1945 to 1985. This evaluation was based on the toxicological models of HAH effects that have been confirmed by numerous laboratories in a variety of species. Again, this study is consistent with toxicological information regarding the effects of HAHs on lake trout and strongly supports the coherence criterion.

Conclusion

The complexity of an ecosystem as large as the Great Lakes makes simple cause-and-effect relationships difficult, if not impossible to demonstrate. A relationship between HAHs and the lack of recruitment observed in lake trout in the lower Great Lakes during the last half of the 20th century is no exception. This chapter has focused on HAHs and other chemicals as potential causative agents in the lake trout reproductive failures in the lower Great Lakes, but it would be naïve to think that factors such as predation by invasive species, genetics, and habitat quality, among others, did not have an influence on lake trout populations during this period. We have presented multiple lines of evidence of biological field information, molecular mechanisms, toxicological models and testing, life history information, and a retrospective risk assessment which, together, suggest that HAHs were causally linked to lake trout recruitment failures in Lake Ontario, Lake Michigan, and likely Lake Huron during the mid- to late 20th century. Ecoepidemiological criteria also support a causal relationship between HAHs in lake trout and their failures to reproduce naturally over this period. Biological factors, such as predation of lake trout by sea lamprey, were important in the initial crashes of adult lake trout populations, and chemical agents such as DDT were likely affecting lake trout recruitment and the ability to reestablish self-sustaining lake trout populations. The major lines of evidence, however, suggest that exposure of lake trout to HAH chemicals with TCDD-like AhR agonist activity was the key causative factor in the recruitment failures observed in populations of lake trout in the lower Great Lakes for nearly four decades of the 20th century.

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22

The Effects of Polycyclic Aromatic Hydrocarbons in Fish from Puget Sound, Washington

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CONTENTS

Introduction

Over the past 20 years, a number of studies have been conducted on the effects of polycyclic aromatic hydrocarbons (PAHs) on marine fish and other marine biota in Puget Sound. The types of studies include (1) sediment and water sampling to determine the concentrations, types, and distribution of PAHs present in the Sound; (2) studies of the uptake, metabolism, and bioaccumulation of PAHs in both fish and the invertebrates that serve as their prey; (3) studies on the biological effects of PAHs on marine organisms, including the role of PAHs in carcinogenesis in marine fish, as well as the impacts of these contaminants on mortality, growth, reproduction, and disease resistance; and (4) modeling efforts to quantify health risks associated with varying degrees of PAH exposure and to estimate the impact of PAH-related changes on survival and reproductive rates on fish populations. In this chapter, we outline what we have learned from these case studies about the risks posed to marine and estuarine fish from PAHs and discuss the application of this knowledge to the regulation and management of these common environmental contaminants.

Background

Characteristics of Puget Sound

Physical and Hydrologic Features

Puget Sound (Figure 22.1) is located in Washington State in the northwestern United States and is the southern-most glacially carved, fjord-like estuary on the west coast of North America (Thomson, 1994). It is a partially mixed estuary composed of several basins and interconnecting channels with an approximate length of 165 km and a highly variable width ranging up to about 10 km. The main basin has depths exceeding 200 m and extends 75 km from the entrance at Admiralty Inlet to The Narrows near the city of Tacoma. Because of Puget Sound's narrow outlet and shelf at Admiralty Inlet, exchange with ocean water is relatively slow; the mean residence time for water in the central basin is about 120 to 140 days, but it can be much longer in isolated inlets and restricted deep basins (Kennish et al., 1998). Consequently, pollutants tend to be retained within the Sound (PSAT, 2002, 2004). Additionally, in a number of areas in Puget Sound, including sites adjacent to larger metropolitan areas, there is persistent water column stratification, with limited mixing of deep and surface layers. With such stratification, chemical contaminants are more likely to be contained within a smaller area and to remain more concentrated than would be the case if the water column were more fully mixed (PSAT, 2002, 2004). Moreover, sediment mass balance studies for Puget Sound show that the Sound is an efficient sediment trap (MacDonald and Crecelius, 1994). Puget Sound receives sediment particles from the river systems that drain the Cascade mountains, and, to a lesser extent, from shoreline erosion. Much of the sediment accumulates as fine-grained sediment in the central Basin (Baker, 1984). About 30% of Puget Sound is depositional, and sedimentation rates are estimated to be in range of 0.05 to 1.2 g/cm^2 per year (Carpenter et al., 1985). The prevalence of fine-grained, depositional sediments in Puget Sound acts to encourage the accumulation and retention of sediment-sorbed organic contaminants such as PAHs within the system.

Puget Sound Fish Populations

Puget Sound serves as the habitat for a number of recreationally and commercially important fish species, including Pacific salmon; forage fish such as Pacific herring, sand lance, and surf smelt; and 39 bottomfish stocks that have supported active fisheries in the past (Palsson, 1997; PSAT, 2002, 2004). Among the historically abundant bottomfish species are spiny dogfish, skates, spotted ratfish, Pacific cod, walleye pollock, Pacific whiting, rockfishes, lingcod, sablefish, greenlings, sculpins, wolf-eel, surfperches, English sole, rock sole, starry flounder, Dover sole, sand sole, and Pacific halibut. In addition to their commercial value, these Puget Sound stocks are of special scientific interest, because they are thought to be evolutionarily younger than related fish stocks along the Pacific coast and may have unique

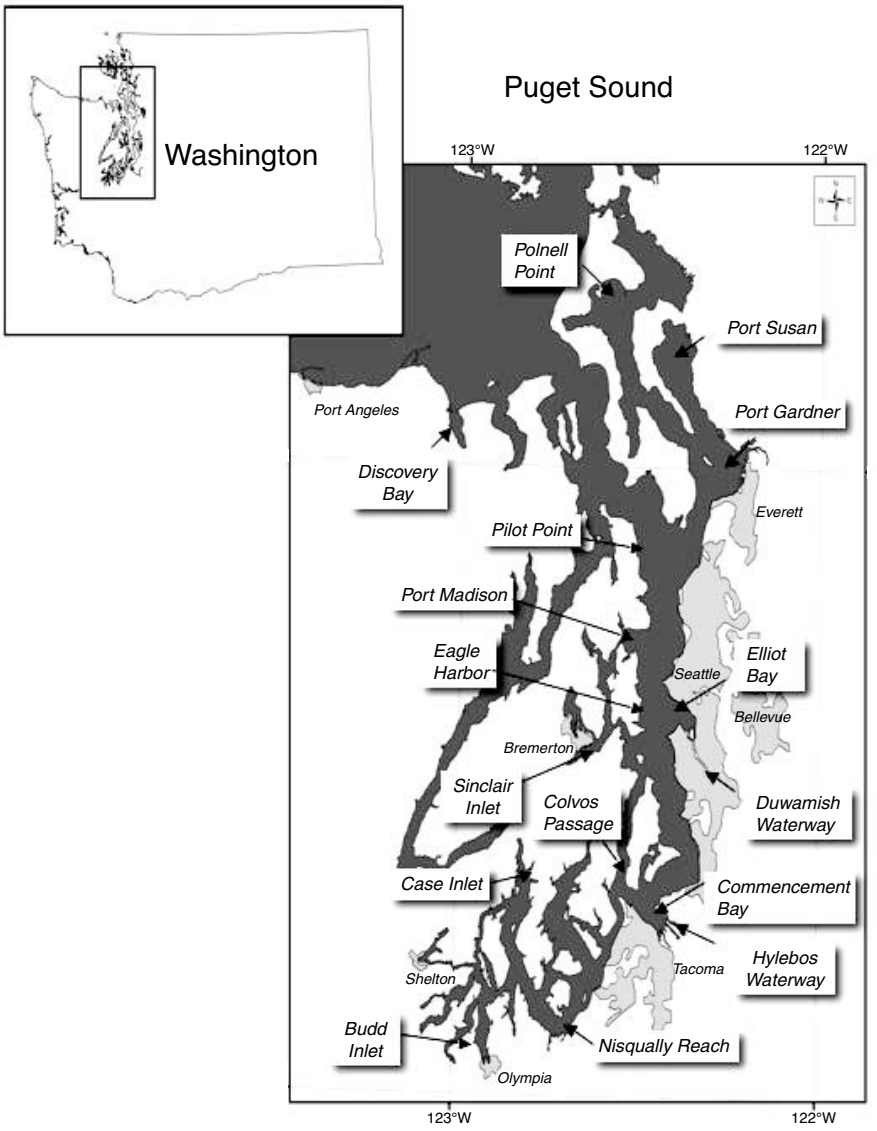

FIGURE 22.1 Map of Puget Sound, Washington, showing locations where sediments and flatfish have been sampled in biomonitoring studies conducted by NOAA Fisheries Northwest Fisheries Science Center and the Washington State Department of Fish and Wildlife.

characteristics that arose during their recolonization of Puget Sound after the last ice age (Rocha-Olivares et al., 1999; Seeb, 1998; Sotka et al., 2005). Much of Puget Sound and the Strait of Juan de Fuca was covered with ice during the maximum extent of the Wisconsin glaciation approximately 15,000 years ago, so genetic bottlenecks and drift could have occurred during recolonization of the Puget Sound fish populations.

Since the mid-1980s, populations of wild salmon, as well as forage fish and bottomfish, have seriously declined, either throughout Puget Sound or in selected embayments in the Puget Sound region (Bargmann, 1988; Palsson, 1997; Schmitt et al., 1994; West, 1997). Puget Sound Chinook and Hood Canal chum salmon have been listed as a threatened species under the Endangered Species Act (ESA) (MacCall and Wainright, 2003; NMFS, 2003). Several marine fish stocks have also been reviewed for ESA listing, including Pacific hake, Pacific cod, Pacific herring, walleye pollock, and brown, quillback, and copper

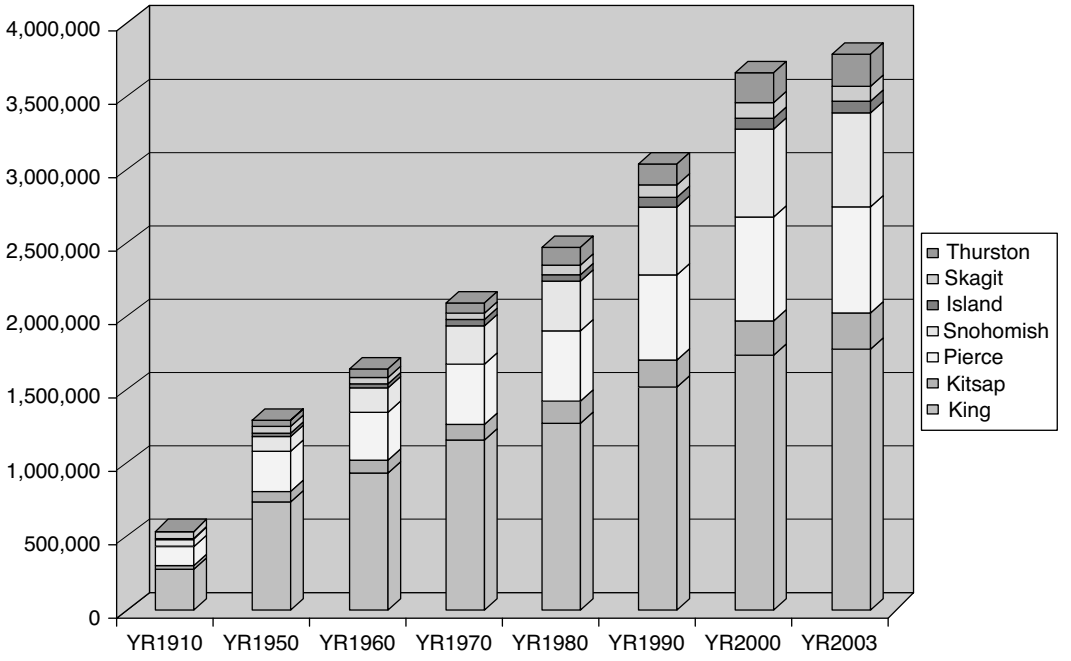

FIGURE 22.2 Population growth in counties bordering on Puget Sound from 1900 to 2003. (Data are from the U.S. Census Bureau, 2006; Puget Sound Regional Council, 2004, 2006.)

rockfish (Gustafson et al., 2000; Stout et al., 2001a,b). It was concluded that, although these species were not currently in sufficient danger of extinction to justify ESA listing, most of them met the International Union for the Conservation of Nature (IUCN) criteria for vulnerable species (Musick et al., 2001). The reasons for the declines of these fish stocks are not clear, but potential contributing factors include overharvesting, natural changes in environmental or climatic conditions, and various types of habitat degradation, including the discharge of toxic chemicals into the marine environment.

Demographics: Human Development

Over the past 100 years substantial urban and industrial development has occurred within the Puget Sound region, resulting in heavy inputs of chemical contaminants at selected sites, as well as significant loss or alteration of marine habitat (Levings and Thom, 1994). According to census data from the State of Washington (Puget Sound Regional Council, 2004, 2006; U.S. Census Bureau, 2006), between 1910 and 1990 the population of the counties bordering on Puget Sound (King, Kitsap, Snohomish, Pierce, Skagit, Island, Thurston, Whatcom, San Juan, Clallam, Jefferson, and Mason counties) increased nearly sixfold (Figure 22.2). Moreover, population growth and related urban and industrial development continued to increase in the Puget Sound region during the 1990s. Populations of Puget Sound counties grew from 13 to 40% between 1990 and 2000, with some of the of the highest increases in historically rural counties. Estimated 2005 populations are 2 to 11% above 2000 levels (U.S. Census Bureau, 2006). The estimated total population in the Puget Sound region area in 2005 was 4.2 million, with 76% residing in King, Pierce, and Snohomish counties, where major urban centers including Seattle, Bellevue, and Tacoma are located. Projected population increases in Puget Sound counties by 2025 range from 20 to 62% (PSAT, 2004). Each of these counties has a major river system and many small stream systems that empty into Puget Sound, and are sources of point and non-point source pollution. Population trends suggest that population growth and increased motor vehicle use in the Puget Sound region will continue, and the geographical area affected by urban development may expand beyond current population centers. These changes are likely to lead to increased and more widespread non-point-source pollution from PAHs in the Puget Sound region.

PAH Contamination in Puget Sound

Sources

Aliphatic, aromatic, and sulfur- and nitrogen-containing hydrocarbons have all been identified in sediments of Puget Sound. The majority of PAHs associated with sediments in Puget Sound as well as at other coastal urban sites originate from petroleum and combustion products (MacDonald and Crecelius, 1994; Varanasi et al., 1992). The geographic distribution of combustion PAHs in Puget Sound suggests that major sources are municipalities and industries that generate large quantities of PAHs, such as aluminum smelting, creosote, and oil refining. Atmospheric emissions from incineration and automobile emissions are other major sources of PAHs. PAHs are also introduced into marine systems through accidental spills of fuel oil, crude oil, and other petroleum products. The suite of PAHs detected in aquatic systems can generally be divided into two broad classes: the lower molecular weight compounds with one to three fused benzene rings (LAHs), which are mainly petrogenic or oil derived, and the higher molecular weight compounds with four to six rings (HAHs), which are mainly pyrogenic or combustion derived (Varanasi et al., 1992). Both classes tend to adsorb to organic or inorganic matter and become immobilized in sediments; however, relative to the LAHs, the HAHs are more hydrophobic and tend to remain more tightly sorbed to sediment, so they are more likely to be trapped in sediments and accumulate in depositional areas of the Sound.

Trends in PAH Concentrations

Studies of Puget Sound sediment cores (Carpenter and Peterson, 1989; Crecelius et al., 1985; MacDonald and Crecelius, 1994) show that, at most sites, maximum PAH concentrations (e.g., ~8 to 12 mg/kg dry wt.) (MacDonald and Crecelius, 1994) occurred between 1945 and 1960, probably as a result of domestic coal burning, which increased until about 1950. Generally, total PAH concentrations appeared to decrease in surface sediments for the next 20 to 30 years, as coal has been gradually replaced by other fossil fuels that produce less PAH (Gschwend and Hites, 1981). Levels reported in sediment cores by MacDonald and Crecelius (1994) were about 4 to 5 mg/kg dry wt; however, this trend is countered by increasing fossil fuel use as a result of the increasing population, as well as increasing urbanization with its associated increased PAH-laden street and stormwater runoff. Consequently, PAH levels in sediments at a number of Puget Sound sites remain well above background concentrations. Recent surveys (EVS, 2003; Long et al., 2003) report sediment PAH levels above Washington State sediment quality standards at a number of sites in Puget Sound.* The majority of these sites were located in urban embayments, including Bellingham Bay, Sinclair Inlet, Everett Harbor, Elliott Bay, and Commencement Bay (EVS, 2003; Long et al., 2003). At some sites in Puget Sound, sediment PAH levels appear to be increasing. A recent study by Washington State Department of Ecology comparing surface sediment collected in 2000 to results from 1989 through 1996 at 10 long-term Puget Sound sites showed that PAH levels were significantly higher in samples collected in 2000 than they were historically at 5 of the 10 sampling stations (EVS, 2003; Long et al., 2003; PSAT, 2004). Total HAH levels were, on the average, 1.5 times higher than they were historically, while total LAH levels were 2.5 times higher.

Co-occurrence with Other Contaminants

Because PAHs are often found at urban sites near industrial discharges, they generally co-occur with a variety of other industrial pollutants, including polychlorinated biphenyls (PCBs), pesticides, and heavy metals, particularly in depositional zones that are distant from point sources. In Puget Sound, strong correlations are found between PAHs and PCBs in sediments from many of the major urban sites, such as the Duwamish Waterway, Elliott Bay, Commencement Bay, and Hylebos Waterway (EVS, 2003; Long et al., 2003). Other studies (Meador et al., 1994) have demonstrated high correlations among PAH compounds and toxic metals, including lead and copper. This feature can make it difficult to separate

* Washington State screening level guidelines for HAHs and LAHs, respectively, are 960 mg/kg total organic carbon (TOC) and 370 mg/kg TOC, or 9.6–19.2 and 3.7–7.4 mg/kg dry wt sediment for typical TOC values of 1 to 2% in Puget Sound sediments (Washington State Department of Ecology, 1995).

the effects of PAHs from those of other compounds through field studies alone. Cause-and-effect relationships between PAHs and disease conditions in fish can be more definitively established through controlled exposure studies in the laboratory; however, even this approach does not fully address the possible modifying effects of other contaminants on the toxicology of PAHs in fish. The additive, synergistic, or antagonistic effects of various compounds in contaminant mixtures are not well understood, and this issue is of continuing concern in establishing exposure limits and sediment quality guidelines for marine organisms.

Exposure to PAHs

Bioavailability

The proportion of the total contaminant concentration that is available for uptake by organisms defines the bioavailable fraction. For neutral hydrophobic organic compounds such as PAHs, organic carbon is the main variable controlling bioavailability. Hydrophobicity, or the tendency to be water insoluble, is the primary determinant for partitioning behavior between water, sediment, and tissue (Burgess et al., 2003). As the hydrophobicity of PAHs increases, the ratio of water to sediment concentrations of a PAH will decline due to the tendency of the compound to avoid water and seek a nonpolar environment. The affinity for tissue also increases with hydrophobicity because of lipid in the organism.

The octanol–water partition coefficient (K_{ow}) is one physical parameter that can be used to predict the partitioning behavior exhibited by PAHs in the environment. A review of several studies was compiled in Meador et al. (1995a) for 24 of the more commonly measured PAHs to provide estimates of their K_{ow} values. The range in K_{ow} values is almost 4 orders of magnitude (about 5000-fold) from the least hydrophobic to the most hydrophobic compound on this list.

Another useful partition coefficient (K_{oc}) is the ratio of PAH concentrations in sediment organic carbon and water, which is determined by the concentration of PAH per gram of organic carbon in sediment divided by the concentration of PAH in water. This coefficient is useful for predicting the amount of waterborne PAH for a given sediment concentration under equilibrium conditions. It has been shown for many neutral hydrophobic compounds that the K_{ow} is a good predictor of the K_{oc}. Several authors have developed equations that predict K_{oc} values from the K_{ow} for various hydrophobic compounds (Di Toro et al., 1991; Karickhoff, 1981; Means et al., 1980). These studies show that the K_{oc} values range from $0.4*K_{ow}$ to $1.0*K_{ow}$.

A few studies have found differential partitioning and bioavailability, depending on the type of PAH and its history of association with sediment. Varanasi et al. (1985), for example, demonstrated that recently added (spiked) PAH in sediment was more bioavailable to organisms than the PAH in field-contaminated sediment; however, other studies have shown only about a twofold reduction in bioaccumulation by infaunal invertebrates exposed to sediment that had been aged several months with contaminants compared to sediment recently spiked with contaminants (Kukkonen and Landrum, 1998; Loonen et al., 1997). The source of the PAH is also a factor in determining bioavailability. Farrington et al. (1983) pointed out that PAHs from oil spills may be less strongly sorbed to sediment than pyrogenic PAHs and hence more available to organisms. This observation was supported by subsequent work showing that PAHs from field samples are tightly bound to sediment and only a few percent is available for equilibrium partitioning (McGroddy et al., 1996; Meador et al., 1995b). The lower water concentrations of PAHs in such sediments can produce much lower than predicted accumulation in organisms; however, it is not clear if species ingesting sediment with pyrogenic PAHs will also exhibit lower bioaccumulation (Meador et al., 1995b).

Bioaccumulation/Food Web Transfer

Bioavailability and organism physiology and behavior are the most important variables affecting the bioaccumulation of chemical contaminants, especially PAHs (Meador, 2003). Of the total environmental concentration, only the bioavailable fraction is available for assimilation into the organism. Unlike

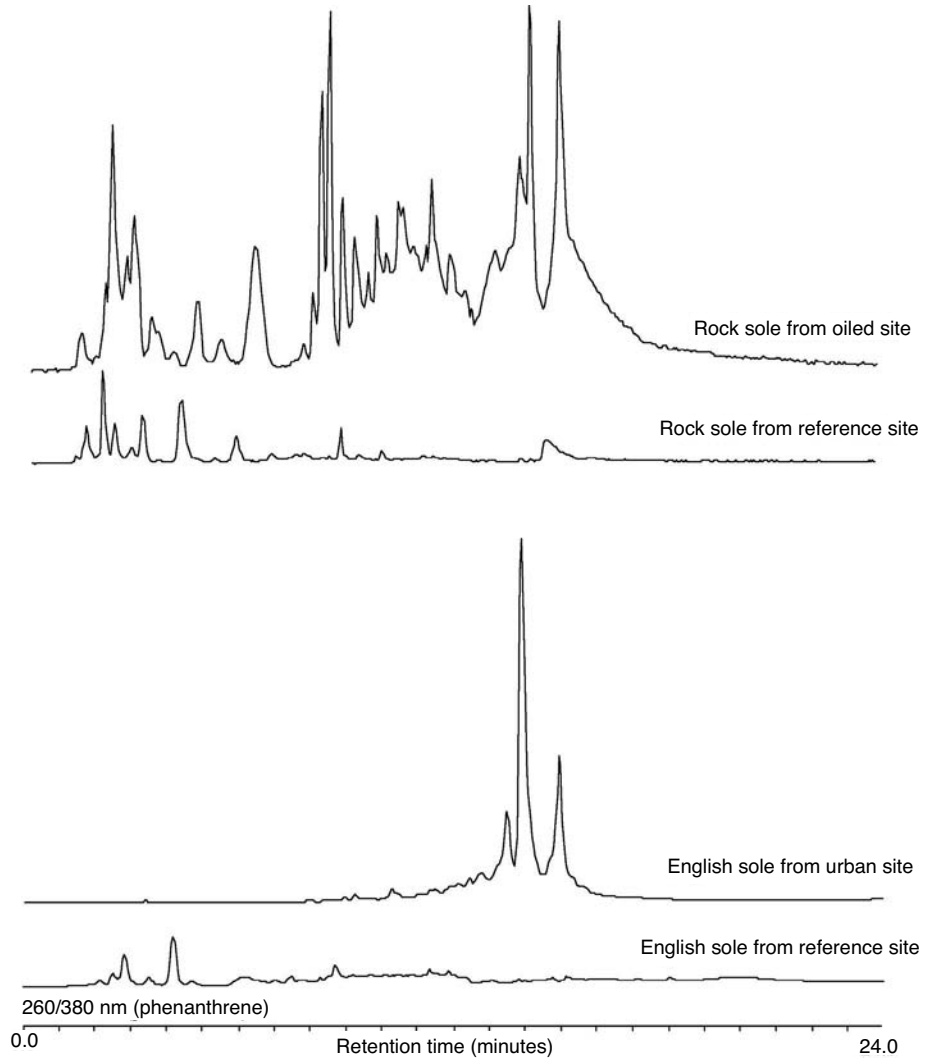

FIGURE 22.3 Chromatograms from the HPLC-fluorescence screening of bile at phenanthrene wavelength: from a rock sole from Prince William Sound, Alaska, after the *Exxon Valdez* oil spill; from a rock sole from a reference (non-oiled) site; and from English sole captured from an urban site and from a non-urban reference site. (From Krahn, M.M. et al., *J. Chromatogr.*, 642, 15–32, 1993. With permission.)

metals and some ionizable organic compounds, the bioavailability of PAHs is affected by only a few environmental variables such as organic carbon and sediment surface area. Physiological factors, including lipid levels and the rates of uptake and elimination (metabolism, diffusion, and excretion), also determine contaminant tissue residues (Meador et al., 1995a). Behavioral patterns, such as organism site fidelity and variable rates of feeding, are very important for determining steady state tissue concentrations.

Bioaccumulation factors for PAHs and other hydrophobic organic compounds are generally expressed as the ratio of tissue to water (BCF) (Equation 22.1) or the sediment concentration ratio (BAF) (Equation 22.2). Currently, many researchers are interested in the biota–sediment accumulation factor (BSAF) (Equation 22.3), which is useful for reducing the variability observed in bioaccumulation. The BSAF is the lipid and organic carbon normalized bioaccumulation factor:

$$\text{BCF} = \frac{[\text{tissue}]}{[\text{water}]} \tag{22.1}$$

$$\text{BAF} = \frac{[\text{tissue}]}{[\text{sediment}]} \tag{22.2}$$

$$\text{BSAF} = \left(\frac{[\text{tissue}]}{f_{lip}}\right) \Big/ \left(\frac{[\text{sediment}]}{f_{oc}}\right) \tag{22.3}$$

where [tissue] and [sediment] are in dry weight (parts per billion [ppb] or parts per million [ppm]), f_{oc} is the dry-weight fraction of organic carbon in sediment (g/g), and f_{lip} is the dry-weight fraction of lipid in tissue (g/g). Several factors, such as variable uptake and elimination rates, reduced bioavailability, and insufficient time for sediment–water partitioning or tissue steady state can affect each of these bioaccumulation factors.

Due to the propensity of hydrophobic compounds, such as PAHs, to partition into lipid, the K_{ow} has also been used as a surrogate measure to predict bioaccumulation. Several authors have developed equations to predict the BCF for a given compound based on its K_{ow} value (Lipnick, 1995; Mackay, 1982). The BAF is a useful measure of the amount of compound accumulated by organisms but is highly variable due to different sediment types and organism capabilities. The BSAF is a necessary refinement of the BAF that accounts for these differences and greatly reduces the observed variability. In general, the theoretical maximum BSAF is approximately one (Di Toro et al., 1991) and the empirical maximum values generally range from 2 to 4 (Boese et al., 1995; USEPA and USACE, 1991) for hydrophobic organic compounds at equilibrium in all phases. Because of the amounts of chemical expected in lipid and organic carbon, it is generally believed that hydrophobic organic compounds that are not metabolized will produce predictable levels of bioaccumulation. Although the BSAF is useful for characterizing bioaccumulation, adjustments have to be made for compounds that are metabolized and when conditions are not at equilibrium.

Equilibrium partitioning (EqP) theory is used to predict the amounts of hydrophobic organic compounds bioaccumulated in organisms (Di Toro et al., 1991; Pavlou and Weston, 1983). The basic premise for EqP is that when sediment and water are in equilibrium, the organism receives an equivalent exposure from each phase allowing predictions of the accumulated dose using either phase. The organismal lipid, total organic carbon (TOC) in sediment, and water can be considered as three phases that exhibit predictable concentrations at equilibrium due to equal chemical activity or fugacity. Because of this assumption, the route of exposure (e.g., water ventilation or prey/sediment ingestion) is immaterial because at equilibrium the concentration in each phase is a function of the thermodynamic properties, not the kinetics of accumulation. Also, because of the equal fugacity between phases, one phase (e.g., sediment) may be used to predict bioaccumulation from all phases, even though the organism may not interact directly with sediment. EqP has generally been successful in predicting sediment–water partitioning (K_{oc}) and bioaccumulation (BSAF) for the nonmetabolized, neutral hydrophobic organic compounds.

Because PAHs are so readily metabolized, predictive models such as the ones mentioned above are rarely accurate in determining PAH bioaccumulation. Potential bioaccumulation of PAHs can be determined with BSAF by setting the BSAF to the maximum (e.g., 4), rearranging the equation, and solving for tissue concentration. Other predictive models may include correlating the PAH metabolites measured in bile (bile FACs) with sediment concentrations or determining the half-life of PAHs in a given fish species and extrapolating the small amounts of parent PAH compounds that can be found in tissue. Stomach content analysis, coupled with uptake efficiency, could also be used to determine apparent bioaccumulation. Whatever methods are employed, it is crucial to be able to gauge the relative amounts of PAHs that are accumulated when attempting to assess exposure and effects at contaminated sites. This is especially true if the goal is to relate an exposure concentration with deleterious effects.

Despite the substantial uptake of PAHs by fish through exposure to food, water, and sediment, rapid metabolism of these compounds by fish precludes high levels in their tissues. Additionally, metabolism of PAHs by invertebrates is variable (James, 1989; Livingstone, 1991; Varanasi et al., 1989a) and can

have a major impact on the amounts that accumulate in fish. Biomagnification of PAHs, which is the increase of contaminants over two trophic levels or more, does not appear to occur in fish (Suedel et al., 1994). Benthic invertebrates in the diet are an important source of PAH exposure to marine fishes, as these compounds bioaccumulate in many invertebrate species. In one study (Varanasi et al., 1992), sediment from the Duwamish Waterway, a contaminated estuary in Puget Sound, was used to examine the uptake of PAHs by clams and amphipods. The HAHs were clearly accumulated by clams and amphipods, but accumulation of LAHs was not as great. Determining the cause of this pattern is complicated because of many factors that must be considered. Although the bioaccumulation factor will be higher in most invertebrates for the HAHs, some species will exhibit metabolism of these compounds, whereas others will not. The preferential retention of HAHs in invertebrates observed in laboratory exposure (Meador et al., 1995b; Varanasi et al., 1992) is also seen in free-living invertebrates and in the stomach contents (e.g., molluscs, arthropods, and annelids) of fish sampled from urban areas (Varanasi et al., 1989a).

Some studies of dietary uptake of PAHs in fish generally indicate low uptake efficiency. Experiments with rainbow trout demonstrated uptake efficiencies between 2 and 30%, depending on the PAH (Niimi and Dookhran, 1989), which was confirmed by another study (Niimi and Palazzo, 1986) with the same species fed herring oil spiked with PAHs, but these results were contrary to those obtained for nonmetabolized hydrophobic organic compounds such as PCBs (Madenjian et al., 1999). These studies on PAHs indicate that uptake efficiency generally declines with increasing chemical hydrophobicity, which may be due to a combination of slow kinetics and a short residency time in the gut. Because the role of metabolism is often not addressed in studies of dietary uptake efficiency, the parent compound plus metabolites must be determined. Recent work indicates uptake efficiencies of approximately 50% for organic compounds with a $\log_{10} K_{ow}$ in the range of 3 to 6.5, which includes most PAHs (Arnot and Gobas, 2004). Additional research that includes uptake and elimination kinetics is needed to better assess uptake efficiency of PAHs for the different routes of uptake, especially the dietary route. These data will help greatly in predicting bioaccumulation from different environmental matrices.

Exposure Assessment Techniques

Because fish metabolize PAHs rapidly (Hellou and Payne, 1986; Roubal et al., 1977; Statham et al., 1978; Varanasi et al., 1989b), measurement of tissue PAH concentrations by standard analytical techniques is generally not a useful method for assessing exposure. Instead, alternative chemical and biological methods have been developed that have become essential tools for evaluating exposure to PAHs in marine fish. For any method, but in particular for a biochemical method, to be useful, it must undergo extensive validation to establish the interpretive limits for the data obtained. This section presents the underlying validation and field studies for three techniques for assessing PAH exposure (bile metabolites, DNA adducts, and CYP1A) that have been applied to Puget Sound fishes.

Bile Metabolites

As mentioned above, exposure to PAHs in fish generally should be assessed by measuring metabolites rather than the parent compounds. Two methods that employ reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection have been developed in our laboratory to screen for PAH metabolites in fish (Krahn et al., 1993). The first, a bile screening method, was developed to evaluate anthropogenic contamination by PAHs in fish sampled from urban estuaries (Krahn et al., 1984, 1986). At the present time, this method or variations of this method are widely used to assess PAH contamination in the environment (Aas and Klungsoyr, 1998; Beyer et al., 1996; Escartin and Porte, 1999a,b,c; Jonsson et al., 2003; Lin et al., 2001; McDonald et al., 1995). In addition, a method that estimates the concentrations of AC metabolites in fish tissues has been developed to address the issue of seafood contamination (Hufnagle et al., 1999; Krone et al., 1992).

In addition to estimating exposure to PAHs, HPLC chromatographic patterns can often provide information about the possible source of contamination, such as crude oil, diesel fuel, or pyrogenic contaminants; for example, the chromatographic patterns from bile of flatfish captured from Prince

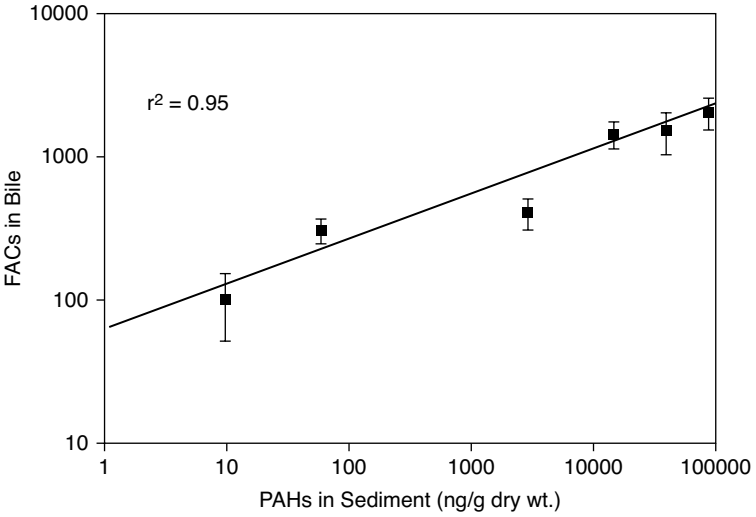

FIGURE 22.4 Relationship between levels of fluorescent aromatic compounds in bile of oyster toadfish and concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediment of the Elizabeth River. (From Collier, T.K. et al., *Environ. Sci.*, 2, 161–177, 1993. With permission.)

William Sound after the *Exxon Valdez* spill are very dissimilar to those of fish exposed to contaminants from urban sites (Figure 22.3). The source of contamination suggested from the HPLC chromatogram can often be substantiated by examining the relative proportions of PAH metabolites determined by gas chromatography–mass spectrophotometer (GC–MS) analysis of bile (Krahn et al., 1987, 1993). Bile of fish exposed to PAHs from urban sites typically contains high proportions of four- to six-ring PAHs from pyrogenic sources, whereas the bile of fish exposed to crude oil contains much larger proportions of metabolites of alkylated naphthalenes, phenanthrenes, and dibenzothiophenes than bile from urban fish.

Laboratory studies have shown that biliary fluorescent aromatic compound (FAC) levels increase in a dose-dependent manner with exposure to PAHs, and field studies have demonstrated strong and consistent correlations between biliary FAC concentrations in fish and sediment PAH concentrations at sites where the animals are collected (Figure 22.4). Biliary FAC concentrations, however, reflect relatively short-term exposure to PAHs. Concentrations typically increase very quickly with exposure, within a day, but decline to baseline levels within about 2 to 4 weeks of exposure (Anulacion et al., 1995; Collier and Varanasi, 1991).

CYP1A Activity

One of the earliest changes associated with exposure to contaminants is induction of cytochrome P450-associated enzymes (CYP) in the liver, especially CYP1A, which is largely responsible for metabolism of PAHs and a variety of other toxic compounds (Buhler and Williams, 1989; Goksøyr and Forlin, 1992). Although several methods are available to assess induction of CYP1A in fish, the most common methods are catalytic assays to measure the functional activity of the enzyme—for example, aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-*O*-deethylase (EROD) activities—and immunoquantitation of the CYP1A protein directly by methods such as an enzyme-linked immunosorbent assay (ELISA). Three of these measures (AHH activity, EROD activity, and CYP1A quantitation by ELISA) were evaluated in Puget Sound flatfish species during a year-long field study (Collier et al., 1995). Each measure could detect significant between-site differences that were consistent with PAH concentrations in sediment where the fish were collected, but AHH activity measured by a standardized protocol showed the least amount of unexplained variability and was the measure most sensitive to site differences. For this reason, in our studies, we have primarily used measurement of hepatic AHH activity for monitoring of CYP1A induction in fish. We believe that this method is particularly useful for analysis of trends in contaminant exposure in fish (Collier et al., 1998a). Because CYP1A is inducible by a wide variety of organic chemical

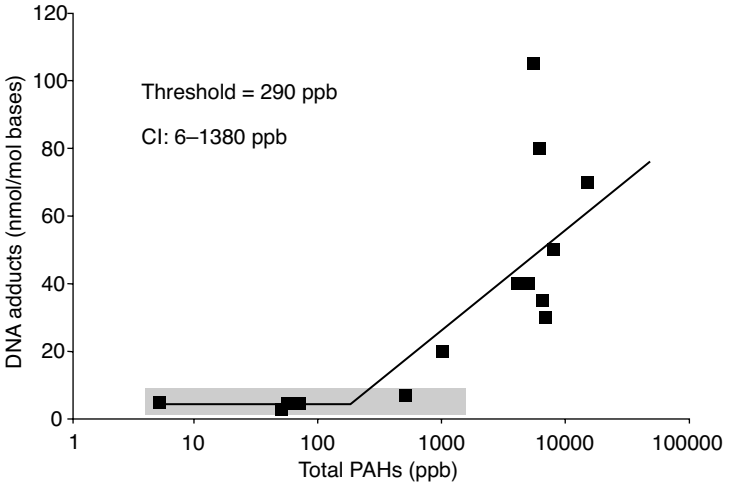

FIGURE 22.5 Relationship between levels of PAH-like hepatic DNA adducts in oyster toadfish and concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediment of the Elizabeth River. (From Collier, T.K. et al., *Environ. Sci.*, 2, 161–177, 1993. With permission.)

contaminants, however, this measure should not be presumed to be diagnostic of PAH exposure in field situations, unless PAHs are the only likely organic contaminant present, such as following oil spills (Collier et al., 1996).

DNA Adducts

Covalent binding of carcinogenic PAHs to DNA (DNA adducts) in liver, an initial molecular step in the chemical hepatocarcinogenesis model (Farber and Sarma, 1987), is observed in several species of fish exposed to benzo(*a*)pyrene (BaP) and related PAHs (Collier et al., 1993; Ericson et al., 1999; Sikka et al., 1991; Varanasi et al., 1989c). A very sensitive technique for determining levels of DNA adducts in fish tissues is the ^{32}P-postlabeling (PPL) method, which was developed in Dr. Kurt Randerath's laboratory in the early 1980s (Gupta et al., 1982) and has evolved substantially since then (Reichert et al., 1999). Currently, the ^{32}P-postlabeling technique is the most sensitive method for the detection of a wide range of bulky, hydrophobic compounds bound to DNA. For hydrophobic, aromatic DNA adducts, such as PAH–DNA adducts, this method can detect 1 adduct in 109 to 1010 (Gupta, 1985; Reddy and Randerath, 1986). The versatility and high sensitivity of the assay has led to the broad use of the ^{32}P-postlabeling assay in studies with mammals and fish for assessing exposure to environmental genotoxins (Balch et al., 1995; Dunn et al., 1987; Liu et al., 1991; Poginsky et al., 1990; Ray et al., 1991; Stein et al., 1992; van der Oost et al., 1994; Varanasi et al., 1989d) and to specific genotoxic compounds, such as BaP and 7H-dibenzo(*c*,*g*)carbazole (Ericson et al., 1999; Randerath et al., 1984; Schurdak et al., 1987; Sikka et al., 1991; Stein et al., 1993; Varanasi et al., 1989c; Watson et al., 1998).

In 1987, we initiated studies using the ^{32}P-postlabeling assay to evaluate exposure of marine fish to environmental carcinogens. These studies have shown that the levels of hepatic DNA adducts in wild fish positively correlate with the concentrations of PAHs present in marine sediments (Collier et al., 1993; Stein et al., 1992) (Figure 22.5). Moreover, laboratory studies with model PAHs and sediment extracts have shown that PAH–DNA adducts formed are persistent and have chromatographic characteristics similar to DNA adducts detected in wild fish (French et al., 1996; Stein et al., 1993; Varanasi et al., 1989d) (Figure 22.6). The study by French et al. (1996) of English sole exposed to a gradient of contaminated sediments showed that the levels of hepatic DNA adducts increased in both a time- and a dose-dependent manner. These findings suggest that the levels of hepatic DNA adducts found in fish tissues could function as indices of cumulative exposure to potentially genotoxic environmental contaminants, such as carcinogenic PAHs. The use of DNA adducts as an exposure index has several important features. First, it is a quantifiable measure of the biologically effective dose reaching a critical target site

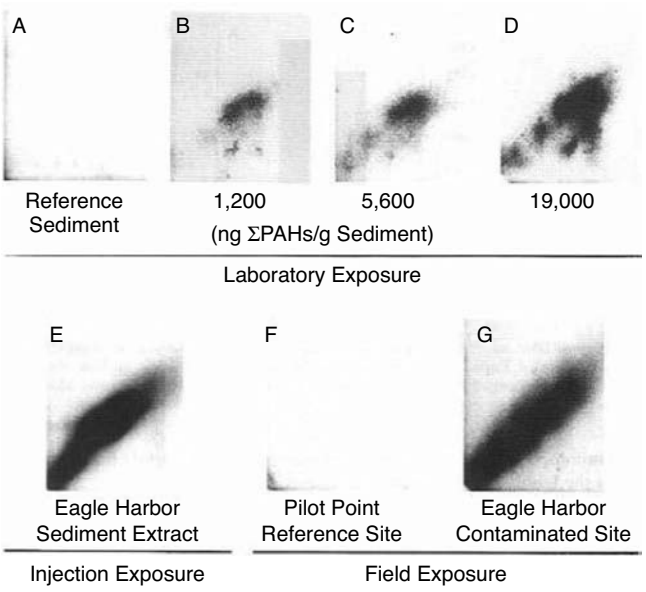

FIGURE 22.6 Computer-generated images of hepatic DNA digests analyzed by the ^{32}P-postlabeling method for English sole exposed for 5 weeks to PAH-contaminated sediments (concentrations of ΣPAHs are (A) 20 ng/g sediment, (B) 1200 ng/g sediment, (C) 5600 ng/g sediment, and (D) 1900 ng/g sediment. Images are also shown for (E) English sole injected intramuscularly with an organic solvent extract of contaminated Eagle Harbor sediment and (G) English sole captured from Eagle Harbor. Hepatic DNA adducts were not observed on images of English sole captured from (F) the reference site, Pilot Point. (From French, B.L. et al., *Aquat. Toxicol.*, 36, 1–16, 1996. With permission.)

and thus is a useful epidemiological/epizootiological tool for detecting exposure to genotoxins. Second, DNA adduct levels integrate multiple toxicokinetic factors (i.e., uptake, metabolism, detoxication, excretion, and covalent binding of reactive metabolites to target tissues). Third, the DNA adduct profiles and levels can be used in identifying species differences in exposure and processing of genotoxic compounds.

Hepatic DNA adducts are currently being used as a marker of exposure to potentially genotoxic contaminants in environmental monitoring of Puget Sound and have been applied in national monitoring programs, such as the National Benthic Surveillance Project of the National Oceanographic and Atmospheric Administration (NOAA) National Status and Trends (NS&T) Program and in the Bioeffects Surveys of NOAA's Coastal Ocean Program. The International Council for the Exploration of the Sea (ICES) has recently published a methods manual on the ^{32}P-postlabeling assay to encourage its use in marine biomonitoring studies (Reichert et al., 1999).

Other investigators have also detected DNA damage in English sole from Puget Sound sites with different assessment techniques. Using GC–MS with selected ion monitoring (GC–MS/SIM) and Fourier-transform infrared (FT–IR) spectroscopy, Malins and colleagues (Malins and Gunselman, 1994; Malins et al., 1996, 1997) have observed hydroxy-radical-induced DNA damage in liver tissue of English sole from contaminated sites in Puget Sound (e.g., the Duwamish Waterway). Common types of damage included hydroxyl-radical-induced ring-opening products (e.g., 2,6-diamino-4-hydroxy-5-formamidopyrimidine) and 8-hydroxy adducts of adenine and guanine (e.g., 8-hydroxyguanine). These mutagenic base modifications were statistically correlated with an increased incidence of preneoplastic or degenerative liver lesions (e.g., basophilic foci, hepatocellular karyomegaly, megalocytic hepatosis, hyalin droplet formation, and apoptosis) in English sole (Malins et al., 1996).

Related forms of oxidative damage have been detected in juvenile salmonids exposed to dietary PAHs in the laboratory (Bravo, 2005). Juvenile rainbow trout (*Oncorhynchus mykiss*) fed a diet containing a mixture of 10 common HAHs at 40 or 400 mg/kg, 160 mg/kg BaP, or 160 mg/kg benzo(*e*)pyrene had induced CYP1A1 in liver and kidney and increased oxidative damage as indicated by DNA strand breaks measured in blood by comet assay, protein nitration measured in kidney by immunohistochemistry, and

lipid peroxidation measured in kidney by F2-isoprostanes. Fish from the highest dose PAH treatment group yielded the strongest effect. After 50 days of exposure to PAHs, fish responses began to moderate but were still significantly different from control. Reactive oxygen species generated in the kidney and liver likely contributed to the damage observed in kidney and blood. The head kidney is thought to be the primary immune organ in fish (Wester et al., 1994), so oxidative stress in this organ might contribute to decreased animal fitness through increased disease susceptibility (Livingstone et al., 2001).

Bioindicators as Integrated Indicators of Toxicant Exposure and Response

In ecotoxicological field studies, we typically employ several of the techniques described above to provide an integrated picture of PAH exposure. All three indicators (bile metabolites, CYP1A induction, and DNA adducts) are strongly correlated with environmental exposure measures, such as concentrations of PAHs in sediments or food, and also show significant correlation with each other. Each of the exposure indicators, however, has its own specificity, sensitivity, and time response; for example, bile FAC concentrations and AHH activity respond relatively quickly to PAH exposure but decline fairly rapidly when exposure ceases, with half-lives of 2 to 4 weeks (Anulacion et al., 1995). In contrast, hydrophobic DNA adducts are relatively stable, with a much longer half life (Anulacion et al., 1995; Stein et al., 1993), so they are better indicators of relatively long-term, cumulative exposure to genotoxic PAHs. The indicators also vary in their specificity; both DNA adducts and biliary FAC concentrations respond specifically to PAH exposure, while CYP1A may be induced in response to either PAHs or coplanar PCBs, dioxins, and related compounds. Moreover, studies indicate that species may differ in their range of response to these indices. For example, in one study with several species of Puget Sound flatfish, AHH and EROD activity in rock sole was substantially higher and more uniform over a range of sediment PAH and PCB concentrations than was the case for either English sole or starry flounder. Consequently, a suite of measurements used concurrently can enhance the ability to identify fish populations affected by exposure to chemical contaminants (Stein et al., 1992).

Biochemical indicators of exposure can also be very useful in epizootiological analyses of field data on PAH exposure and disease conditions in fish. Risk factors for certain diseases or other forms of biological injury can be generated by correlating biochemical indicators of PAH exposure with the disease occurrence, thus allowing the use of a relatively simple biomarker in predicting risk. Additionally, because these parameters can be measured in individual fish, factors such as age and sex can be taken into account in risk analyses. Several examples of such analyses are discussed in the section on PAH exposure and biological effects in Puget Sound fish.

These biochemical parameters can also yield important information on the uptake and metabolism of PAHs and provide insight into mechanisms of toxicant action and pathogenesis of toxicopathic hepatic lesions and other disease conditions in fish. As an example, immunohistochemical localization of CYP1A activity, in combination with quantitation of PAH–DNA adducts, has been applied to investigate the role of resistance to cytotoxicity in liver neoplasia in English sole (Myers et al., 1998a). Immunohistochemical studies of English sole from PAH-contaminated sites show a consistent reduction in expression of CYP1A in hepatic neoplasms and most preneoplastic foci of cellular alteration. The reduction in CYP1A expression is accompanied by a significant and nearly parallel reduction in DNA adduct level as compared to non-neoplastic liver tissue. These findings are consistent with the hypothesis, developed from studies with mice and rats, that neoplastic hepatocytes possess a resistant phenotype in which there is a reduced capacity to activate PAHs and related compounds to toxic and carcinogenic intermediates (Roomi et al., 1985).

Similarly, the presence of PAH–DNA adducts in tissues of PAH-exposed marine fish suggests the potential for genomic alterations, including oncogene activation. Our laboratory has now cloned and sequenced the entire K-*ras b* cDNA from liver of English sole (Peck-Miller et al., 1998). The percent identity between the predicted amino acid sequence of English sole and human K-*ras b* was 97%, whereas the percent identity between the English sole gene and rainbow trout or rivulus K-*ras b* was 98%. Areas of amino-acid sequence conservation include codons 12, 13, and 61, the positions in which mutations are observed in *ras* cellular oncogenes in other species. Analysis of K-*ras* mutations was performed on a variety of necrotic, preneoplastic, and neoplastic lesions in livers from 13 English sole

collected from contaminated waterways in Puget Sound; however, despite reports of K-*ras* mutations in hepatic tumors from other fish (Chang et al., 1991; McMahon et al., 1990; Wirgen et al., 1989), no mutations in codons 12, 13, or 61 were found in hepatic neoplasms and related lesions from English sole by direct DNA sequencing of polymerase chain reaction-amplified genomic DNA. The results suggest that K-*ras* has a role in liver carcinogenesis that varies according to the fish species or carcinogen. Future studies of the etiology of chemically induced cancer in wild English sole should consider mutations in other cancer-related genes, such as *p53*, which is involved in the control of apoptosis, or programmed cell death (Fridman and Lowe, 2003), as well as Ha-*ras* and N-*ras*, which may have oncogenic activities similar to those of K-*ras* (Crespo and Leon, 2000). Forms of all three of these genes have been identified in other fish species (Bhaskaran et al., 1999; Brzuzan et al., 2006; Busch et al., 2004; Cachot et al., 2004; Franklin et al., 2000; Rotchell et al., 2001).

PAH Exposure and Biological Effects in Puget Sound Fishes

General Approach to Ecotoxicological Investigations

We have addressed the impact of PAHs and related industrial pollutants on benthic fish in Puget Sound through a broad interdisciplinary approach. This research has involved exposure assessment tightly linked to the detection of effects at several levels of biological organization. Exposure assessment is typically carried out by measurement of PAHs in sediments at sites where fish are collected, as well as in stomach contents of target fish species, and through measurement of PAH metabolites in fish bile. Animals are also examined for early biochemical changes, such as CYP1A induction and DNA adducts, which can often be linked in a dose-responsive fashion to PAH exposure, both in the field and in the laboratory through controlled experiments. Additionally, fish are examined for effects of contaminants on critical life processes, such as growth, reproduction, and immunocompetence and disease susceptibility, which may then be related to potential changes in vital rates and fish abundance. Our primary target species in Puget Sound is English sole (*Parophrys vetulus*). This pleuronectid species was selected because it is widespread, and in early studies (Malins et al., 1984, 1985) it appeared to be particularly sensitive to the effects of chemical contaminants. We have sampled English sole at a range of both urban and non-urban embayments throughout Puget Sound (Figure 22.1). These sites vary widely in sediment PAH concentrations (Figure 22.7). In addition to English sole, we have examined PAH exposure and effects in several other Puget Sound bottomfish (e.g., rock sole and starry flounder), as well as in several species of juvenile salmon that use estuaries during their migration from fresh to saltwater.

Field Assessment of PAH Exposure in Puget Sound Fish

Bottomfish

Field assessments of PAH exposure in English sole and other bottomfish have generally included measurement of biliary FACs, CYP1A induction, and DNA adducts. This suite of indicators is useful in evaluating both long- and short-term exposure to PAHs, with the caveat that CYP1A induction, while responsive to PAH exposure, is not diagnostic of PAH exposure. Of the three indicators, biliary FACs are most purely measures of recent exposure, while the others are indicators, at least to some degree, of biological or biochemical response. Bile screening for PAH metabolites was originally developed as a tool to rapidly estimate concentrations of metabolites resulting from the uptake and transformation of PAHs by English sole (Krahn et al., 1984, 1986). Since that time, the technique has been applied in a number of studies with Puget Sound fish (Collier et al., 1998a,b; Krahn et al., 1987; Myers et al., 1994, 1995, 1998b,c, 2000, 2005; O'Neill et al., 1999; Stein et al., 1992), demonstrating clearly that bottomfish from urban and industrialized areas in Puget Sound take up PAHs. Average biliary FAC concentrations of English sole from representative sites are shown in Figure 22.8. In studies within Puget Sound and in other areas of the United States (Collier et al., 1993; Johnson et al., 1993; Myers et al., 1994; Stehr et al., 1997, 2004), strong and consistent correlations have been found between biliary FAC levels in sole and other bottomfish and sediment PAH concentrations.

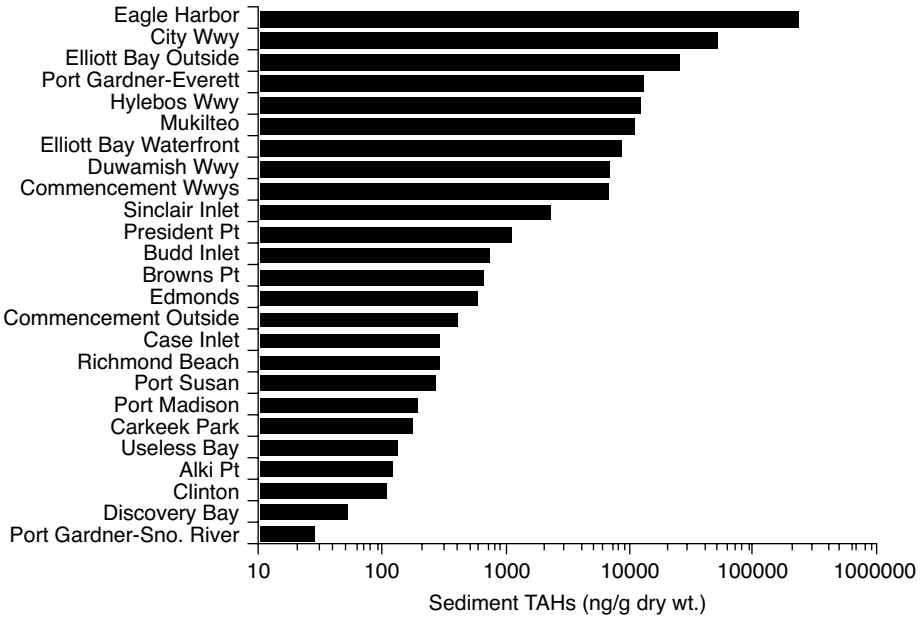

FIGURE 22.7 Concentrations of aromatic hydrocarbons (ng/g dry weight) in sediments from sites in Puget Sound. (From Johnson, L.L. et al., *Can. Tech. Rep. Fish. Aquat. Sci.*, 1948, 304–329, 1994. With permission.)

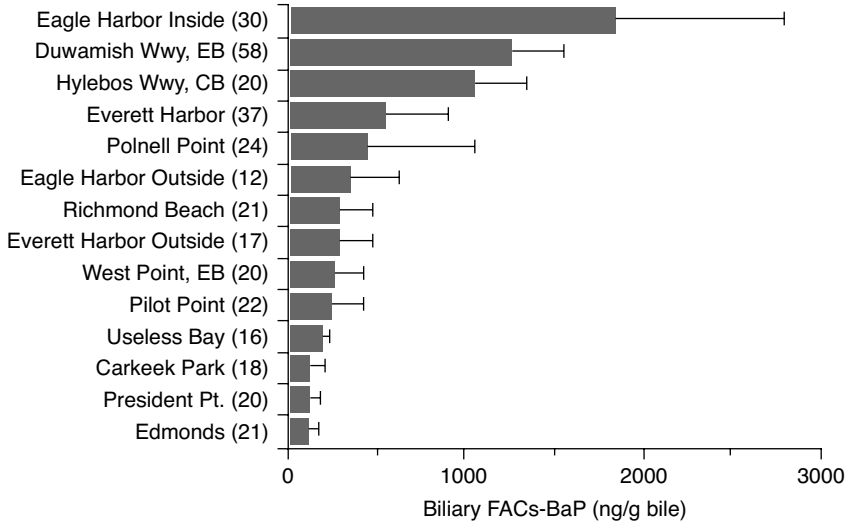

FIGURE 22.8 Concentrations of fluorescent aromatic compounds (FACs) in bile (ng/g bile) of English sole from Puget Sound. (From Johnson, L.L. et al., *Can. Tech. Rep. Fish. Aquat. Sci.*, 1948, 304–329, 1994. With permission.)

Fish from contaminated areas show not only elevated levels of FACs in bile but also other physiological changes that are indicators of biological responses to contaminant exposure, including increased AHH activity and covalent binding of PAHs to DNA in liver. Both of these types of biochemical alteration are observed in English sole, starry flounder (*Platichthys stellatus*), and rock sole (*Pleuronectes bilineata*) from areas in Puget Sound with elevated sediment PAH concentrations (Collier and Varanasi, 1991; Collier et al., 1995; Stein et al., 1992) (Figure 22.9). Moreover, both hepatic AHH activity and levels of DNA adducts in liver, determined by ^{32}P-postlabeling, are significantly correlated with sediment PAH concentrations at sites where fish are collected, and with biliary FAC concentrations in the same fish (Stein et

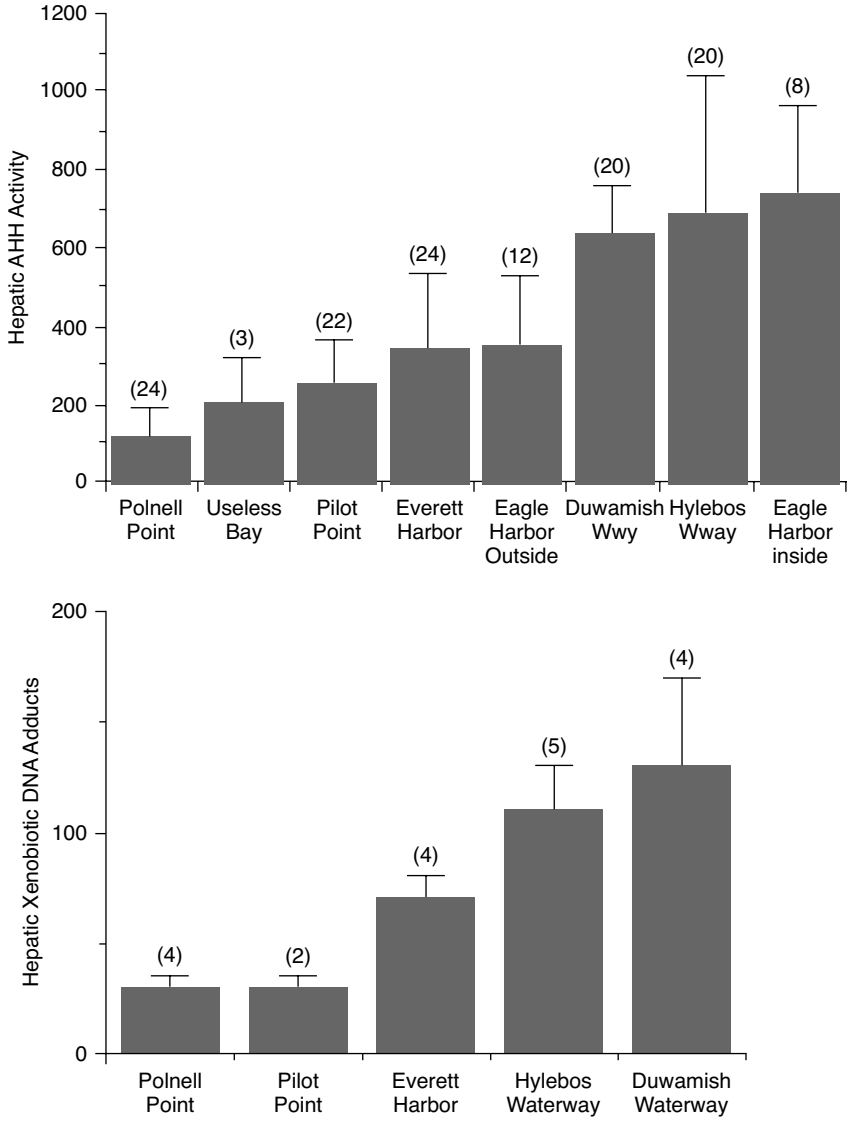

FIGURE 22.9 Hepatic AHH activity and concentrations of xenobiotic–DNA adducts in English sole from four sites in Puget Sound with varying levels of sediment contamination. (From Johnson, L.L. et al., *Can. Tech. Rep. Fish. Aquat. Sci.*, 1948, 304–329, 1994. With permission.)

al., 1992). Laboratory studies suggest that English sole is comparatively less sensitive to CYP1A induction following PCB exposure than to other bottomfish species (Collier and Varanasi, 1991). This finding raises the possibility that the extensive CYP1A induction in English sole from Puget Sound is due primarily to PAH exposure; however, this hypothesis remains unproven. The correlation of FACs with hepatic DNA adducts is consistent with laboratory studies in which PAHs extracted from sediment and model PAHs produced adduct patterns in exposed sole similar to those found in wild sole (Reichert et al., 1998).

Salmon

In addition to benthic fish, such as English sole, which reside in bottom sediments, transient species such as outmigrant juvenile salmon show detectable exposure to PAHs as they migrate through urban estuaries (McCain et al., 1990; Olson et al., 2006; Stehr et al., 2000; Stein et al., 1995). McCain et al.

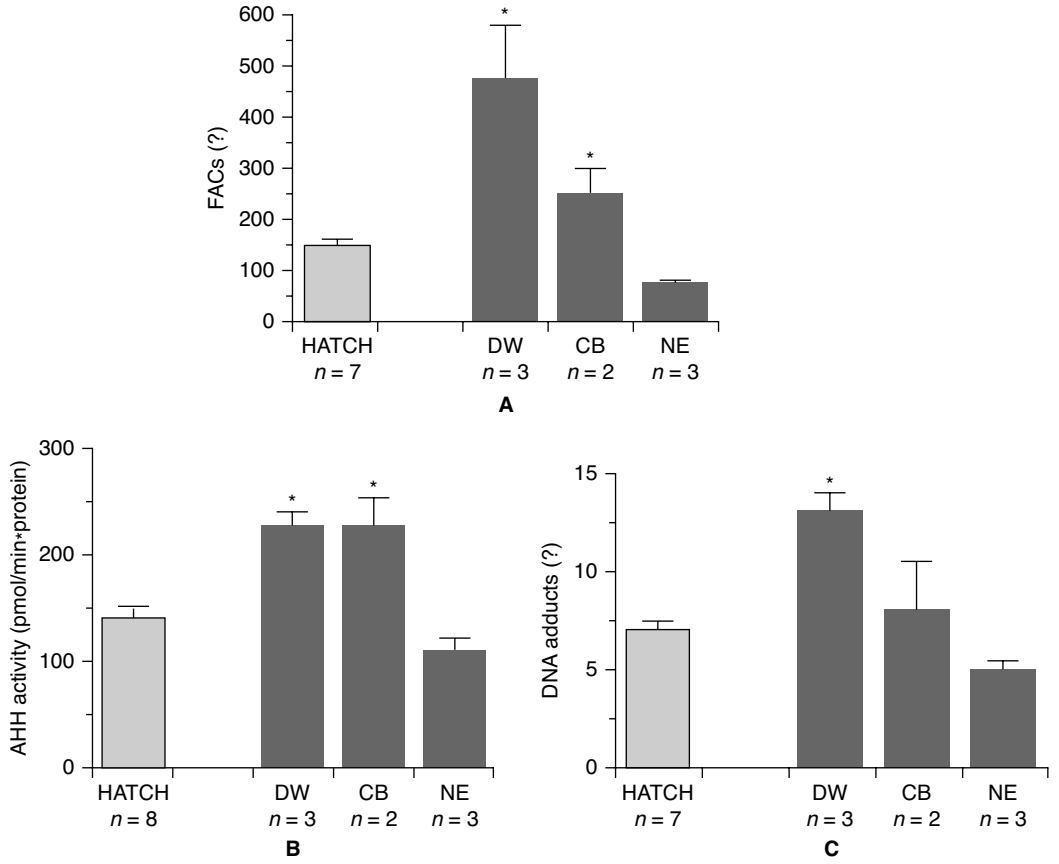

FIGURE 22.10 (A) Biliary fluorescent aromatic compounds (FACs) (ng BaP equiv per mg protein); (B) hepatic aryl hydrocarbon hydroxylase (AHH) activity (pmol/min*mg protein); and (C) hepatic DNA adducts (nmol/mol bases) in juvenile salmon from estuaries and hatcheries sampled from Puget Sound, Washington. The bars represent the mean ± standard error. The sample size (*n*) equals the number of tissue composites that were analyzed. CB, Commencement Bay; DW, Duwamish Waterway; NE, Nisqually River Estuary. (Figure is reprinted from Johnson, L.L. et al., *Can. Tech. Rep. Fish. Aquat. Sci*., 1948, 304–329, 1994. With permission.)

(1990) found significantly higher AH concentrations in stomach contents as well as higher levels of FACs in bile in juvenile Chinook salmon from the Duwamish Waterway compared to juvenile Chinook from the relatively uncontaminated Nisqually Estuary or from either of the hatcheries from which fish in these two river systems are released. Since this initial study, PAH exposure has been examined in juvenile Chinook, Coho, and chum salmon from hatcheries and their respective estuaries of five river systems of Puget Sound: the Green-Duwamish, the Puyallup-Hylebos/Commencement Bay, the Nisqually, the Snohomish, and the Skokomish (Olson et al., 2007; Stehr et al., 2000; Stein et al., 1995). As in the earlier study, salmon collected from the Duwamish and Commencement Bay/Hylebos Waterway estuaries adjacent to Seattle and Tacoma showed elevated levels of FACs in bile in comparison to fish from hatcheries or from the less-urbanized Nisqually and Skokomish systems. Additionally, elevated AHH activity and elevated levels of DNA adducts in liver were detected in salmon migrating through the urban estuaries (Figure 22.10). In comparison to English sole collected from the same waterways, FAC concentrations in bile of salmon were similar. In contrast, levels of DNA adducts in liver of salmon, whose residence time in the estuary is relatively short, were much lower than levels in English sole (Collier et al., 1998b; Stehr et al., 2000). This is consistent with data from dose–response experiments showing that biliary FAC concentrations are a good indicator of relative short-term exposure to PAHs, while DNA adducts are a better measure of chronic, long-term exposure.

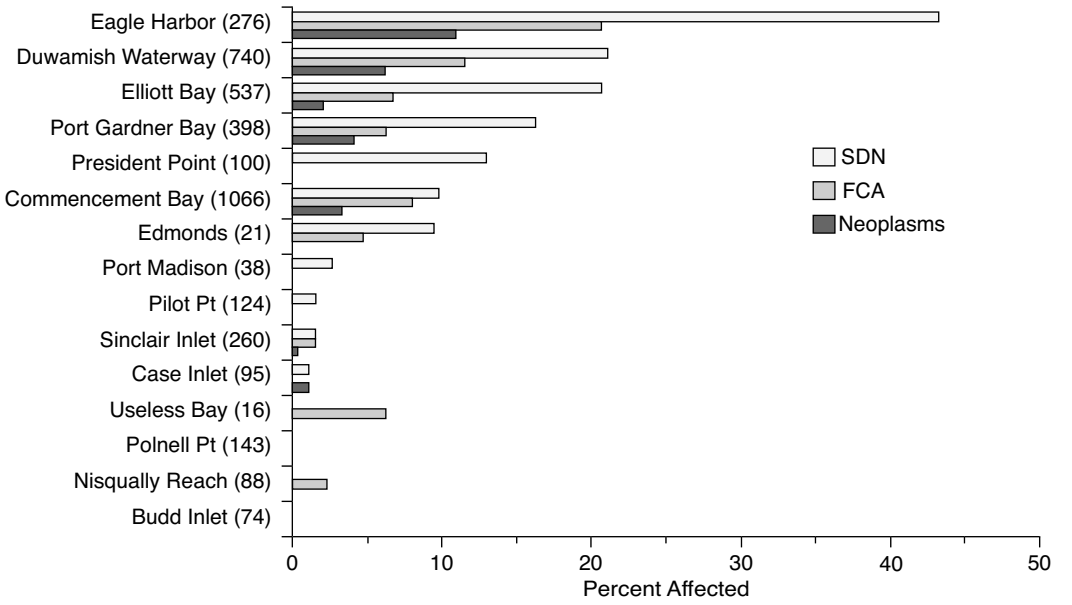

FIGURE 22.11 Prevalences of hepatic lesions in English sole from sites in Puget Sound. FCA, foci of cellular alteration; SDN, specific degeneration/necrosis (megalocytic hepatosis and/or nuclear pleomorphism). The number of animals sampled is indicated in parentheses following the site name. (Data were compiled from Krahn et al., 1984; Malins et al., 1984, 1987; Myers et al., 1987; Stein et al., 1992. Figure is reprinted from Johnson, L.L. et al., *Can. Tech. Rep. Fish. Aquat. Sci.*, 1948, 304–329, 1994. With permission.)

Physiological Effects in Puget Sound Fish

Liver Cancer and Related Toxicopathic Lesions

Liver cancer (and the occurrence of a spectrum of other toxicopathic lesions involved in the pathogenesis of liver cancer) is one of the most dramatic and best-documented effects of contaminants on English sole in Puget Sound (Myers et al., 1987, 1990, 1994, 1998b,c, 2003; O'Neill et al., 1999; PSAT 2002, 2004). In general, the prevalence of these liver lesions increases with increased urbanization (Figure 22.11). Typically, between 25 and 40% of English sole sampled from urban embayments such as Elliott Bay and Commencement Bay exhibit neoplastic, preneoplastic, or unique degenerative liver lesions, as compared to 3 to 8% of fish from non-urban and moderately urbanized sites.

In statistical analyses of field data (Landahl et al., 1990; Myers et al., 1990, 1994, 1998b,c, 2003; Rhodes et al., 1987), exposure to PAHs has been identified as the major risk factor for neoplasms and related liver lesions in English sole, although certain chlorinated hydrocarbons (CHs), including PCBs and pesticides, may also play a role in lesion progression through their actions as tumor promoters. Multiple field studies in bottomfish species, including English sole, have consistently shown strong statistical correlations between both site sediment PAH concentrations and prevalences of toxicopathic hepatic lesions (Myers et al., 1998b,c). A cause-and-effect relationship between PAHs and toxicopathic liver lesions in English sole has been confirmed by induction of degenerative, proliferative, and preneoplastic lesions, identical to those observed in field-collected fish, in sole exposed in the laboratory to model carcinogenic PAHs such as BaP and to extracts of sediments from PAH-contaminated sites (e.g., Eagle Harbor) in Puget Sound (Schiewe et al., 1991).

Field studies have also examined the association between rapidly responding biomarkers of PAH exposure (bile FAC levels, CYP1A induction, and DNA adduct formation) and toxicopathic hepatic lesion prevalences in wild fish. Relationships between liver disease risk and these bioindicators of PAH exposure were clarified in a multi-season study targeting subadult, reproductively immature English sole from nine sites in Puget Sound representing a broad gradient for sediment contaminants (Myers et al., 1998b). This study expanded upon information provided in companion studies examining links between

liver histopathology and a more limited set of biochemical markers in subadult English sole, rock sole, and starry flounder at all seasonal sampling points (Myers et al., 1992) and relationships among the various biochemical markers of contaminant exposure in English sole, rock sole, and starry flounder in specimens of these species sampled at a single time point (Stein et al., 1992).

Although neoplasms were rarely detected in these subadult fish, whose average age was less than 2 years, higher prevalences of the earlier-occurring lesions were found in young sole from contaminated sites. Prevalences for any of the earlier-occurring lesions were significantly higher at all of the moderately to severely contaminated sites (Duwamish Waterway, inside Eagle Harbor, inside Everett Harbor, and Commencement Bay). Confirming earlier results in adult English sole (Myers et al., 1990, 1994, 1998c), biliary FAC levels were significant risk factors for prevalences of these early lesion categories in logistic regression analyses. More interestingly, the study also identified induction of hepatic AHH activity as a significant risk factor for prevalences of all of the early lesion categories, and DNA adduct level as a significant risk factor for nuclear pleomorphism/hepatic megalocytosis as well as the inclusive early lesion category.

With the acquisition of additional data on lesion occurrence, DNA adduct levels, and AHH activity in wild fish, it has been possible to establish hepatic DNA adducts as a significant risk factor for hepatic lesion occurrence on an individual fish basis. In stepwise logistic regression analyses that accounted for the potential influences of fish age and sex on the risk of lesion occurrence, hepatic DNA adduct level was identified as a positively significant ($p \leq 0.05$) risk factor for the occurrence of hepatocellular nuclear pleomorphism and hepatic megalocytosis in English sole, starry flounder, and rock sole and for preneoplastic foci of cellular alteration in English sole (Myers et al., 1998b,c). These findings give further credence to the role of PAH exposure in the etiology of these lesions and suggest that xenobiotic–DNA adduct formation is integral to the early initiation phase of hepatocarcinogenesis, as well as the induction of these more prevalent lesions known to occur prior to the development of hepatic neoplasms in fish and rodent models of hepatocarcinogenesis. Moreover, they support the utility of certain non-neoplastic hepatic lesions as early indicators of biological damage in subadult and adult fish environmentally exposed to xenobiotics. Overall, the collective available evidence shows a strong causal relationship between PAH exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (Myers et al., 2003).

Reproductive Effects

In addition to toxicopathic liver disease, English sole residing in contaminated areas in Puget Sound also suffer from various types of reproductive impairment. Field studies conducted in the late 1980s (Johnson et al., 1988) showed that female English sole from areas with high concentrations of PAHs in sediment were less likely to enter vitellogenesis and had lower plasma concentrations of the female reproductive hormone 17β-estradiol than sole with low levels of contaminant exposure (Figure 22.12). At minimally to moderately contaminated sites within Puget Sound such as Port Susan and Sinclair Inlet, approximately 80 to 90% of adult females underwent gonadal development, while at the Duwamish Waterway and Eagle Harbor the percentage declined to 40 to 60%. A 1994 field study of English sole from the Hylebos Waterway in Commencement Bay, a site with high sediment concentrations of CHs and PAHs, yielded similar results (Johnson et al., 1999). Approximately 55% of adult female sole from the Hylebos Waterway were vitellogenic, in comparison to 80% of adult sole from Colvos Passage, a nearby reference site. As in the earlier study, exposure to PAHs was a major risk factor for inhibited ovarian development in adult sole. English sole from PAH-contaminated areas also display increased ovarian atresia, particularly of primary oocytes (Johnson et al., 1988, 1997). Increased atresia is associated with a trend toward reduced egg production and increased egg weight, although the magnitude of the effect is not great. Similarly, in gravid female dolly varden and yellowfin sole sampled following the *Exxon Valdez* oil spill, plasma estradiol concentrations were depressed in fish with high biliary FAC levels (Sol et al., 2000).

Results of these field studies are supported by laboratory experiments showing that pretreatment of gravid female English sole with extracts of contaminated sediment or crude oil containing high levels of PAHs decreased levels of endogenous estradiol (Johnson et al., 1995; Stein et al., 1991). Related experiments suggest that exposure to BaP- or PAH-contaminated sediment may suppress estradiol-induced vitellogenin production in English sole (Anulacion et al., 1997).

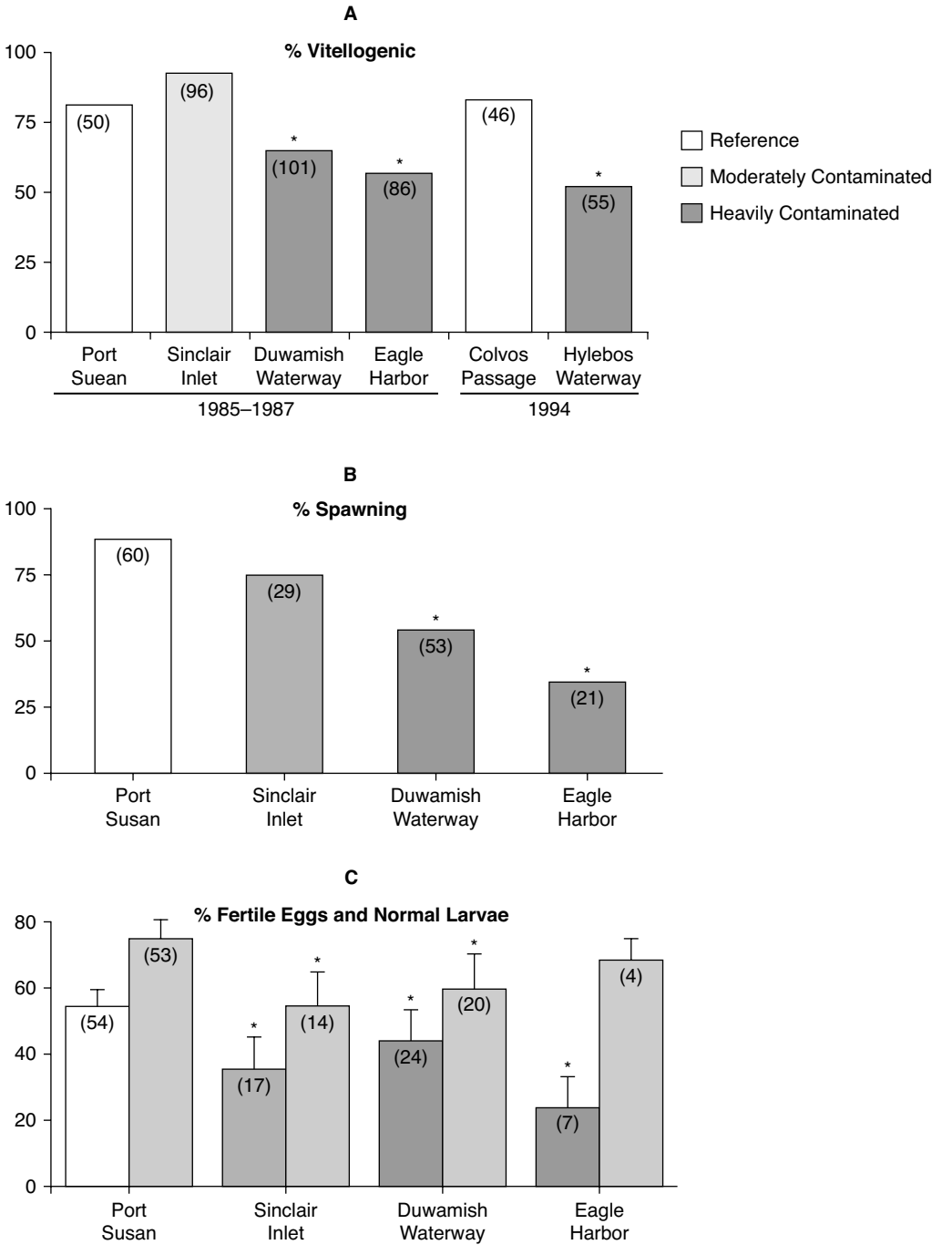

FIGURE 22.12 Reproductive success of English sole from urban and non-urban sites in Puget Sound. Shown in the figure are (A) the percentage of adult female sole entering vitellogenesis; (B) the percentage of adult female sole successfully spawning following induction with luteinizing hormone releasing hormone analog; and (C) the percentages of fertile eggs (solid) and normal larvae (diagonal lines) produced by those females that spawned successfully, following fertilization with pooled sperm from reference male English sole. The number of animals sampled at each site is indicated in parentheses. Values significantly different from those at the reference sites (Port Susan for 1985–1987 studies and Colvos Passage for 1994 study) are indicated by asterisks (*). (Data were compiled from Casillas et al., 1991; Collier et al., 1997; Johnson et al., 1988. Figure is reprinted from Johnson, L.L. et al., *J. Sea Res*., 39, 125–137, 1998. With permission.)

Studies also suggest that English sole from contaminated areas that do successfully enter vitellogenesis may experience inhibited spawning ability and reduced viability of eggs and larvae. When gravid English sole from Port Susan, Sinclair Inlet, the Duwamish Waterway, and Eagle Harbor were brought into the laboratory and artificially induced to spawn, spawning success was significantly lower in fish from Eagle Harbor and the Duwamish Waterway, where sediment PAH concentrations are high (Casillas et al., 1991). Exposure to PAHs in the water column (e.g., fluoranthene at 0.075 to 7.5 mg of PAH per L seawater) caused larvae to become disoriented and to exhibit signs of narcosis, with mortality at higher concentrations (Eddy et al., 1993).

Although effects of PAHs on reproduction have not been studied as extensively in male as in female English sole, evidence suggests that males may also be susceptible to PAH-related reproductive dysfunction. Preliminary studies indicate that, although testicular development in male sole from PAH contaminated sites is relatively normal, plasma concentrations of 11-ketotestosterone and testosterone are reduced in fish with particularly high concentrations of PAH metabolites in bile (Sol et al., 1998).

The impacts of PAHs on the reproduction and development of wild Puget Sound salmon have not been well characterized, although some laboratory exposure studies have shown developmental abnormalities in Pacific Northwest salmon species exposed to PAHs (Ostrander et al., 1988, 1989). Also, the effects of PAHs on early development were investigated extensively in salmon and other fish after the 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska. Following the *Exxon Valdez* spill, field and laboratory studies in several species, including Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*), demonstrated a common syndrome of oil-induced embryolarval toxicity characterized by pericardial and yolk sac edema, jaw reductions, and curvature of the body axis (Carls et al., 1999; Couillard, 2002; Heintz et al., 1999; Marty et al., 1997; Pollino and Holdway, 2002), generally resulting in embryo death. Delayed mortality also occurred in fish with no external malformations, as indicated by the reduced oceanic survival of pink salmon exposed to weathered crude oil as embryos and released as smolts (Heintz et al., 2000). Although the mechanisms leading to PAH-associated malformations and sublethal effects during fish development were not fully understood, it was assumed that toxicity was mediated largely through PAH interactions with the aryl hydrocarbon receptor (AhR) (Nebert et al., 2004; Schmidt and Bradfield, 1996), producing a syndrome similar to that associated with exposure to other potent AhR ligands such as dioxin (Peterson et al., 1993). More recently, Incardona et al. (2004, 2005) have found that, although the dioxin-like syndrome may occur with exposure to certain high-molecular-weight PAHs such as pyrene, exposure to the lower molecular weight tricyclic PAHs that are the most common components of weathered crude oil results in an alternative toxic response, cardiotoxicity, which is AhR independent. This cardiovascular dysfunction appears to be a major cause of deformities associated with exposure to petrogenic PAHs such as those in weathered crude oil. These studies raise the possibility that lower exposure concentrations, within the range often found in the environment, may cause subtle cardiovascular effects in fish that otherwise appear normal. This might explain, for example, the reduced marine survival of pink salmon exposed as embryos to lower levels of weathered Alaska North Slope crude oil from the *Exxon Valdez* spill (Heintz et al., 2000). Similar deformities could also be affecting survival of Pacific herring and Pacific Northwest salmon in Puget Sound, but this has not yet been confirmed with field studies. This work highlights the importance of sublethal, potentially long-term effects of PAHs that enter the environment through oil spills, as well as other sources (Peterson et al., 2003).

Immunological Alterations

Several studies conducted over the last ten years suggest that fish from PAH-contaminated sites in Puget Sound may have reduced immune function and increased susceptibility to infectious disease. To date, the most extensive research on the effects of PAHs on immune function has been conducted with juvenile salmon. As discussed earlier, juvenile Chinook salmon from polluted waterways in Puget Sound, such as the Duwamish and Hylebos Waterways, show exposure to PAHs as demonstrated by elevated concentrations of PAH metabolites in bile, increased levels of DNA adducts in liver, elevated PAH concentrations in stomach contents, and induction of hepatic CYP1A activity (McCain et al., 1990; Stehr et al., 2000; Stein et al., 1995). In a series of field and laboratory studies, Arkoosh and co-workers (Arkoosh and Collier, 2002; Arkoosh et al., 1991, 1994, 1998, 2001) demonstrated that the immunocompetence

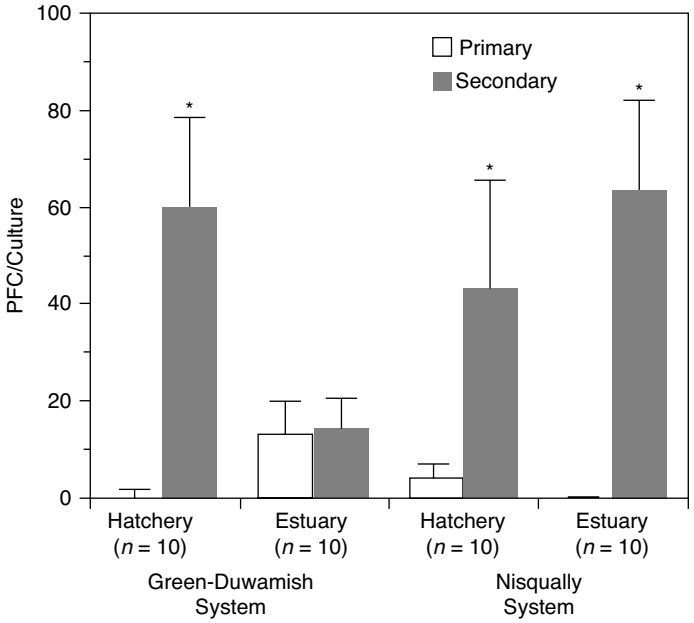

FIGURE 22.13 The mean number ($x \pm$ SD) of primary and secondary plaque forming cells/culture to an antigen for juvenile Chinook salmon from the releasing hatcheries, a nonurban estuary and an urban estuary. (Adapted from Arkoosh, M.R. et al., *Fish Shellfish Immunol*., 1, 261–277, 1991.)

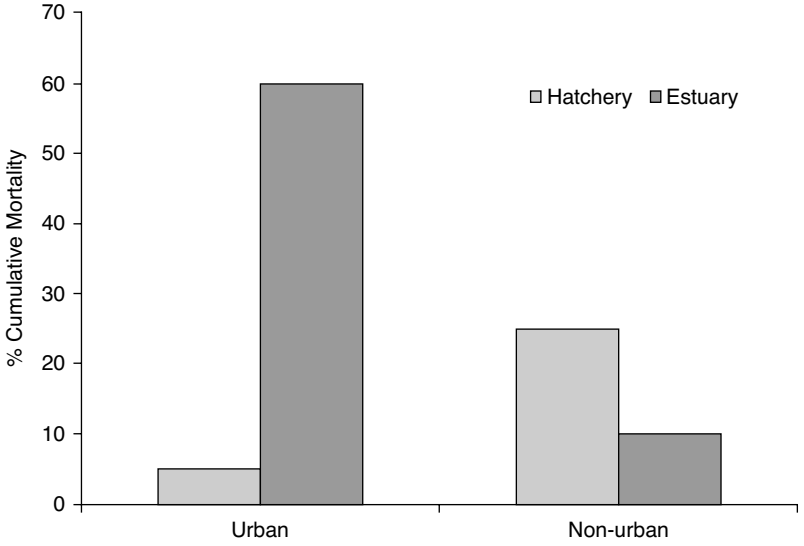

FIGURE 22.14 Percent cumulative mortality of juvenile Chinook salmon from an urban and nonurban estuary and their corresponding hatcheries four days after exposure to the marine pathogen *V. anguillarum*. (Adapted from Arkoosh, M.R. et al., *Trans. Am. Fish. Soc*., 127, 360–374, 1998.)

of juvenile salmon from the Duwamish Waterway was suppressed when compared to salmon from a nonurban estuary or hatcheries (Figure 22.13). Leucocytes of salmon from the urban estuary were unable to generate a secondary (or memory) *in vitro* B-cell immune response following exposure to either the T-cell-independent antigen trinitrophenylated lipopolysaccharide (TNP-LPS) or the T-cell-dependent antigen TNP-keyhole limpet hemocyanin (TNP-KLH), suggesting that PAH exposure suppresses immunological memory (Arkoosh et al., 1991).

To determine if salmon from a contaminated environment are also more susceptible to an infectious disease, we collected juvenile fall-run Chinook salmon from an urban estuary and from a nonurban estuary and the respective releasing hatcheries upstream from these estuaries, and we exposed them in the laboratory to the marine bacterial pathogen *Listonella anguillarum*. We found that juvenile Chinook salmon from the contaminated estuary were more susceptible to *L. anguillarum*-induced mortality than were fish from the corresponding hatchery upstream from the estuary, which were not exposed to contaminants. In contrast, juvenile fall Chinook salmon from a nonurban estuary showed no increase in susceptibility to *L. anguillarum*-induced mortality compared to fish from the corresponding hatchery (Figure 22.14). These disease challenge studies indicated that juvenile Chinook salmon with contaminant-associated immunodysfunction were also more susceptible to one of their natural pathogens (Arkoosh et al., 1998). Follow-up laboratory exposure studies with sediment extracts and contaminant model mixtures determined that contaminants, apart from other estuarine variables specifically associated with the Duwamish and Hylebos Waterways, could independently suppress immune function and increase disease susceptibility in juvenile Chinook salmon (Arkoosh et al., 1994, 2001). Because these fish were exposed to mixtures of contaminants, there was some uncertainty regarding the relative contributions of PAHs and other chemicals present in sediment (e.g., PCBs) to the observed reductions in disease resistance. In a recent disease challenge study with *L. anguillarum*, Palm et al. (2003) found little indication of reduced disease resistance in juvenile Chinook salmon exposed to dietary PAHs at environmentally relevant concentrations; however, these studies were conducted in freshwater, in spite of the fact that *L. anguillarum* is a saltwater pathogen, so the results may not reflect the virulence of *L. anguillarum* in the natural environment.

To better characterize the effects of PAHs on immunocompetence, we exposed juvenile rainbow trout (*Oncorhynchus mykiss*) to PAHs in their diet at environmentally relevant concentrations, similar to those found in stomach contents of Chinook salmon (*Oncorhynchus tshawytscha*) from contaminated estuaries in Puget Sound (Arkoosh et al., 1998), and examined changes in disease resistance and expression of immune-regulating genes (Bravo, 2005). Like salmon in earlier experiments, these fish exhibited higher susceptibility to the pathogen, *Aeromonas salmonicida*, than fish fed the control diet. When we profiled gene expression in head kidney using microarrays, we found that over 20 immunologically relevant genes were differentially expressed after pathogen challenge. Transcripts from five immune genes—interleukin 8 (IL-8), transport associated protein 1 (TAP1), NF-κB essential modulator (NEMO), recombination activating gene 2 (RAG 2), and a major histocompatibility complex II (MHC II) gene—were also measured by RT–PCR at the time points examined by microarray. These genes participate in innate and adaptive immunity and some are key regulators of immune response such as NF-κB modulator (Mann et al., 2001). Moreover, they have been previously been described as important components of resistance of salmon to *A. salmonicida* (Gerwick et al., 2002; Vanya et al., 2005). All selected genes were significantly down regulated by 2 days post-challenge, suggesting that PAH exposure decreases transcription of genes involved in the immune response.

Evidence also suggests altered immune function in English sole exposed to PAHs in the field, PAH-contaminated sediment, or PAH-contaminated sediment extracts from Eagle Harbor (Arkoosh et al., 1996; Clemons et al., 2000) (Figure 22.15 and Figure 22.16). Eagle Harbor, the site of a former creosote plant, is located at Bainbridge Island in Puget Sound and has sediments characterized by high levels of PAHs. In sole exposed to PAHs, the leukoproliferative (mitogenic) response and macrophage production of cytotoxic reactive oxygen intermediates were augmented. In other species, increased production of reactive oxygen species by macrophages has been associated with an increase in peroxidative damage of kidney and gill tissues (Bravo, 2005; Fatima et al., 2000), but the effects of augmented reactive oxygen intermediate production and leukoproliferative response on English sole's immune function are unknown. Studies are currently underway to better establish the linkage between these changes and disease susceptibility in English sole.

Growth

Studies over several years with juvenile Chinook salmon from the Duwamish and Hylebos Waterways suggest that exposure to PAHs may suppress growth in this species (Casillas et al., 1995, 1998a). Growth was monitored in juvenile salmon collected from these sites and held in the laboratory for 90 days, and it was found that growth rates for the fish from urban estuaries were lower than those for fish from the

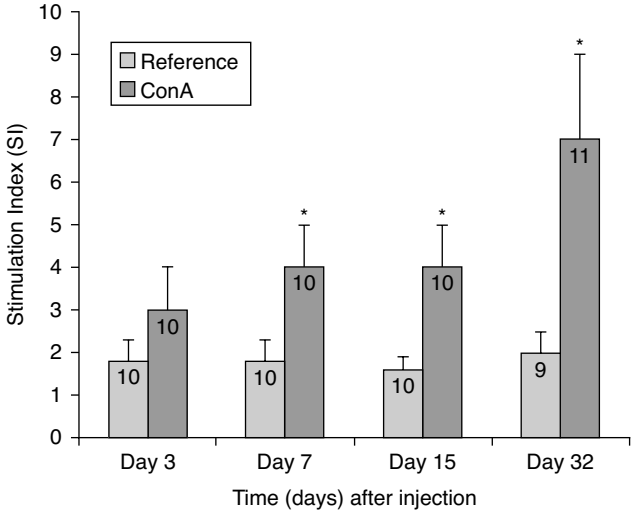

FIGURE 22.15 Stimulation indices of splenic leukocytes from English sole injected with Eagle Harbor Sediment extract (■) or with the carrier acetone/Emulphor EL-620 (□). The LP response of the sole to the mitogen, concanavalin A, was determined on days 3, 7, 15, and 32 after injection. The stimulation index was determined for each individual fish. The asterisk (*) signifies that the EHSE-injected fish produced a significantly different leukoproliferative response than that produced by fish injected with acetone/Emulphor EL-620. The vertical bars represent 1 SE above and below the mean. Mean tritium disintegrations per minute (±SE) of splenocytes from control cultures (without mitogen) ranged from 130 ± 20 to 520 ± 200. (Adapted from Clemons, E. et al., *Mar. Env. Res*., 47, 71–87, 2000.)

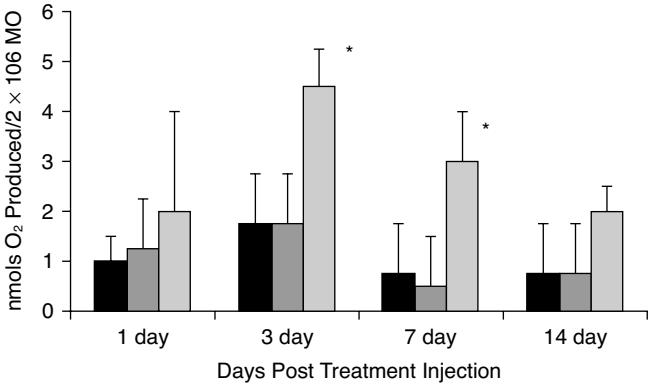

FIGURE 22.16 Amounts of superoxide produced (mean + SD) by elicited peritoneal macrophages from uninjected English sole (■), sole injected with acetone/Emulphor EL-620 (■), or sole injected with sediment extract (□) stimulated with opsonized zymosan (OZ) *in vitro* at 1, 3, 7, and 15 days after exposure to PAHs. Six fish per treatment per day are represented, except at day 3 there are 5 fish from the sediment-extract-injected group and at day 14 each treatment group is represented by 7 individuals. The asterisk (*) signifies that the sediment-extract-injected group is significantly different ($p < 0.05$) from either the uninjected group or the group injected with acetone/Emulphor EL-620. (Adapted from Clemons, E. et al., *Mar. Env. Res*., 47, 71–87, 2000).

corresponding hatcheries or from nonurban estuaries. Furthermore, concentrations of plasma hormones involved in the regulation of growth in fish, such as thyroxine (T_4), triiodothyronine (T_3), and insulin-like growth factor (IGF), were altered in salmon from urban estuaries in comparison with hormone levels in hatchery or non-urban fish (Casillas et al., 1995, 1998a, unpublished data). These findings suggest that exposure to PAHs and other organic contaminants may interfere with the endocrine modulation of growth in juvenile salmon, resulting in impaired overall growth.

Laboratory exposure experiments with model compounds and sediment extracts from contaminated Puget Sound sites also indicate that exposure to PAHs may suppress growth or alter the metabolism of

juvenile salmon (Casillas et al., 1998a,b; Meador et al., 2006). There is some uncertainty in these and other studies regarding the concentrations of PAHs required to suppress growth of juvenile salmon if fish are exposed to PAHs alone. In studies by Casillas et al. (1998a,b), fish exposed to PAHs alone at concentrations comparable to those present in the Hylebos Waterway did not exhibit consistent reductions in growth in all treatment groups, although growth was reduced consistently in fish exposed to sediment extracts containing PAHs in combination with PCBs and other contaminants. In a feeding study, Palm et al. (2003) found no effects on the growth of juvenile Chinook salmon exposed to PAHs for 28 days at a concentrations as high as 252 mg/kg dry wt. (or approximately 50 mg/kg wet wt.) feed; however, the lipid content of the diet was substantially higher than would be expected in a natural diet, and this may have made the detection of alterations in growth rate more difficult. More recently, Meador et al. (2006) conducted a dietary feeding study in which juvenile Chinook salmon were dosed with PAH at concentrations in feed ranging from 1 to 1171 mg/kg dry wt. (or approximately 0.2 to 234 mg/kg wet wt.), a range encompassing PAH concentrations measured in stomach contents of juvenile salmon from Pacific Northwest estuaries (Johnson et al., 2007; Stehr et al., 2000; Stein et al., 1995; Varanasi et al., 1993). Significant differences in mean fish weight did not appear except in fish at the two highest doses, but at the lowest dose (38 mg/kg dry wt.) variability in fish weight increased significantly. Significant changes were also observed in plasma chemistry and fatty acid profiles of the fish at doses in the range of 122 to 324 mg/kg dry wt.

These studies indicate effects of PAHs on fish growth and energy balance but also suggest that other compounds present in contaminated Puget Sound estuaries, such as PCBs, are contributing significantly to growth reductions that have been observed in field collected fish; however, more work is needed to determine the relative importance of various compounds in generating this effect. The stage of development at which fish are exposed to PAHs may also be important in determining effects, as more definitive reductions in growth have been observed in pink salmon exposed to low levels of crude-oil-associated PAHs during embryonic development, possibly as a result of impaired cardiac function (Heintz et al., 2000; Incardona et al., 2004, 2005).

English sole growth also appears to be affected by exposure to PAHs. Data from two laboratory studies (Kubin, 1997; Rice et al., 2000) show reduced growth in juvenile English sole exposed to PAHs through contaminated sediments or diet. Kubin (1997; see also Johnson et al., 1998, 2002) exposed juvenile English sole to sediments contaminated with PAHs at concentrations of approximately 5 mg/kg, 2.5 mg/kg, and <5 µg/kg dry wt. for 6 months.* For the first 3 months, growth rates were similar for all treatments (1.0 to 1.1% per day for weight and 0.36 to 0.38% per day for length), but during the next 3 months growth rates were significantly lower in the high-exposure group. The percent change in weight was 0.35% per day in fish exposed to 5 mg/kg of PAHs, as compared to 0.43% in control fish; the percent change in length was 0.13% per day in exposed fish as compared to 0.16% per day in control fish. The fish exposed to sediments with total PAH concentrations of 2.5 mg/kg dry wt. showed no significant decrease in growth rate relative to control fish. Actual threshold effect concentrations could be lower, however, because uptake of PAHs was from sediment and water only, and in the natural environment substantial exposure would also occur through the diet via ingestion of invertebrate prey species residing in contaminated sediment.

A study by Rice et al. (2000) confirmed both the effect of PAHs on growth of juvenile English sole and the importance of dietary exposure. The findings showed significantly reduced weight in juvenile English sole fed polychaete worms reared on sediments containing 3 to 4 mg/kg dry wt. PAH after an exposure period of only 28 days. The percent change in weight was markedly less (0.05 to 0.1% per day) in exposed fish, as compared to control fish (1.1 to 1.2% per day). These effects have not yet been corroborated in wild populations of English sole, but if they do occur they could impact sole populations by reducing fecundity or altering the time required to reach sexual maturity (Brandt et al., 1992). Slow growth rates have also been associated with increased juvenile mortality in several fish species (McGurk, 1996).

* Sediment PAH concentrations for this study were determined using the HPLC/PDA screening system of Krahn et al. (1991), which provides an estimate of PAH concentration by measurement of fluorescent aromatic compounds (FACs) in sediments. To validate the screening method, total PAH concentrations in sediment samples from a variety of urban and non-urban sites along the U.S. west coast were analyzed using both HPLC/fluorescence and GC–MS (Krahn et al., 1988).

TABLE 22.1

Associations between PAH Concentrations in Sediments, Stomach Contents, and Bile with Site-Specific Prevalences of Selected Categories of Idiopathic Liver Lesions in English Sole, as Determined by Logistic Regression ($p < 0.05$)[a]

Chemical Class/ Compartment	*n*	*p*-Value/% Total Variance				
		Neoplasms	FCA	SDN	Prolif	Necrosis
ΣLAHs						
Sediment	22	0.020/54	0.001/52	0.001/45	0.001/68	NS
Stomach contents	9	NS	0.001/65	0.001/75	0.001/73	NS
Bile (FACs-L)	20	0.032/15	0.001/26	0.001/35	0.002/21	NS
ΣHAHs						
Sediment	22	0.004/48	0.001/40	0.001/38	0.003/35	NS
Stomach contents	9	NS	0.001/65	0.001/75	0.001/73	NS
Bile (FACs-H)	20	NS	0.01/13	0.001/22	0.001/22	NS

[a] Analyses were performed while adjusting for mean age and sex ratio (female:male). Table indicates *p*-value, and percent of total variance in lesions prevalence explained by risk factor (reduction in scaled deviance).

Abbreviations: FACs-H, aromatic compounds fluorescing at benzo(*a*)pyrene wavelengths; FACs-L, aromatic compounds fluorescing at naphthalene wavelengths; FCA, foci of cellular alteration; HAHs, high-molecular-weight polycyclic aromatic hydrocarbons; LAHs, low-molecular-weight polycyclic aromatic hydrocarbons; *n*, number of sites; NS, not significant; Prolif, proliferative lesions; SDN, specific degenerative/necrotic lesions.

Source: Adapted from Myers, M.S. et al., *Environ. Health Perspect.*, 102, 200–215, 1994.

Risk Characterization

Ecological risk assessment and characterization are critical components of our research program on the effects of PAHs on marine and estuarine fish. Suter (1993) defined risk assessment as "the process of assigning magnitudes and probabilities to adverse effects of human activities or natural catastrophes." This process involves identifying hazards, such as releases of toxic chemicals to surface waters that support fisheries, and uses measurement, testing, and mathematical or statistical models to quantify the relationship between perceived hazards and subsequent adverse health effects. The standard paradigm for ecological risk assessment (NRC, 1983) includes four major steps: (1) hazard definition, which involves the choice of endpoints for the assessment (e.g., mortality, cancer risk, reductions in fecundity) and the target species of interest, environmental description, and source terms; (2) exposure assessment and effects assessment; (3) risk characterization; and (4) risk management.

For the case study illustrated in this chapter, PAHs in the marine environment are the hazard of interest, the primary target species is English sole, and exposure and effects assessment data have already been presented for several endpoints, including hepatic lesions, reproductive dysfunction, and growth. This section on risk characterization describes our efforts to use these data to estimate the types of adverse health effects that would be expected to result from a given environmental exposure to PAHs. Uncertainties in the analysis are discussed, but risk management options are not examined in detail.

The analytical methods we used in the effects assessment and risk characterization steps are primarily logistic regression techniques. These methods calculate the risk of an adverse health effect (e.g., the risk of cancer) associated with a given PAH exposure in an individual fish or fish subpopulation (e.g., fish collected from a particular sampling site). We have used this approach in several studies with English sole and other Pacific Coast bottomfish to statistically relate lesion prevalences to biological risk factors and measures of contaminant exposure (Johnson et al., 1993; Myers et al., 1994; Stehr et al., 1997, 1998, 2004). Through stepwise logistic regression techniques, it is possible to determine the influence of contaminant exposure-related risk factors on hepatic disease risk while simultaneously accounting for the influence of biological risk factors such as age and sex. This technique is commonly used on binomial or proportional data in epidemiological and epizootiological studies (Breslow and Day, 1980).

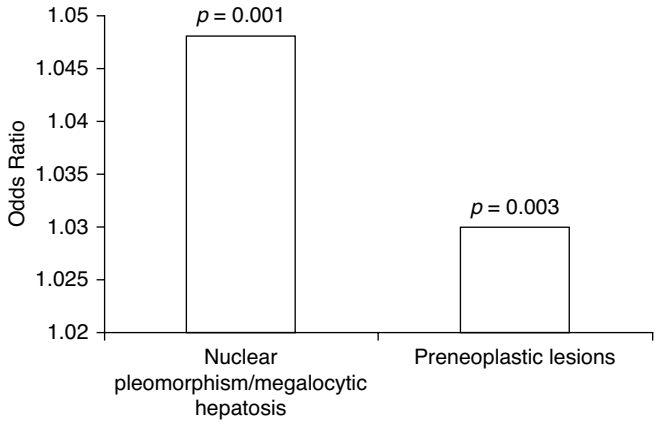

FIGURE 22.17 Risk of hepatic lesion occurrence in English sole (*Pleuronectes vetulus*) from Eagle Harbor attributable to the levels of hepatic polycyclic aromatic compound (PAC)–DNA adducts. The odds ratio for the occurrence of a lesion is an estimation of the degree of association between a risk factor and lesions occurrence and estimates the relative risk for a lesion that is attributable to that risk factor. It is calculated from the variable coefficients of the logistic regression. Odds ratios greater than 1 indicate an increased probability of lesion occurrence for each nmol of DNA adducts present; for example, in the case of the lesion nuclear pleomorphism/megalocytic hepatosis, each additional nmol of DNA adducts in the liver of an individual fish increases the probability of occurrence of that lesion by 1.05 times. (From Reichert, W.L. et al., *Mutat. Res*., 411, 215–225, 1998. With permission.)

Analyses of a number of different datasets (Myers et al., 2003) have consistently identified PAH exposure as a significant risk factor for the development of hepatic lesions in English sole, as well as other marine bottomfish species. Measures of exposure utilized in these analyses include sediment PAH concentrations, PAH concentrations in stomach contents, biliary FAC concentrations, hepatic AHH activity, and levels of DNA adducts in liver. Of the hepatic lesions observed in bottomfish sampled in these studies, preneoplastic focal lesions, proliferative lesions, specific degeneration and necrosis, and hydropic vacuolation were most commonly associated with exposure to and uptake and metabolism of PAHs. In these analyses, we determined the significance of the relationships between prevalences of lesions at particular sampling sites and discrete risk factors such as levels of PAHs in sediments and fish tissues, while adjusting for mean fish age and sex ratio, with each sampling event for a species at a site treated as an independent occurrence. Separate analyses for each contaminant class or chemical-associated risk factor (e.g., PAHs in stomach contents, bile FACs) were performed, with results expressed as the proportion of variation in lesion prevalence attributable to significant risk factors. For example, the data in Table 22.1 show that 35% of the variation in site-specific prevalences for specific degeneration and necrosis in English sole can be explained by mean levels of bile FACs in fish from those sampling sites. Similar logistic regression analyses (Anderson et al., 1980; Schlesselman, 1982) that examine the risk of lesion occurrence associated with a PAH exposure parameter (e.g., DNA adducts as measure of longer term PAH exposure) in individual fish (while accounting for fish age) are capable of generating data and dose–response curves that estimate the relative risk (as expressed by the odds ratio) of liver lesion occurrence attributable to that PAH-exposure parameter. As an example, the odds of an English sole from Eagle Harbor exhibiting specific degeneration or necrosis (also called hepatocellular nuclear pleomorphism/hepatic megalocytosis in pathological terminology) or preneoplastic focal lesions increase by 1.05 and 1.03 times, respectively, for each unit increase in hepatic DNA adduct level (Figure 22.17).

More recent studies have focused on linking adverse fish health effects with sediment PAH concentrations to supply risk characterization information in a form that is more directly applicable to the development of sediment quality guidelines. By applying segmented regression techniques to data from field surveys conducted over the past 15 years in Puget Sound and the west coast of the United States, we estimated the threshold sediment concentrations of PAHs at which increased toxicopathic liver disease and levels of PAH–DNA adducts are initially observed (Horness et al., 1998; Johnson et al., 2002).

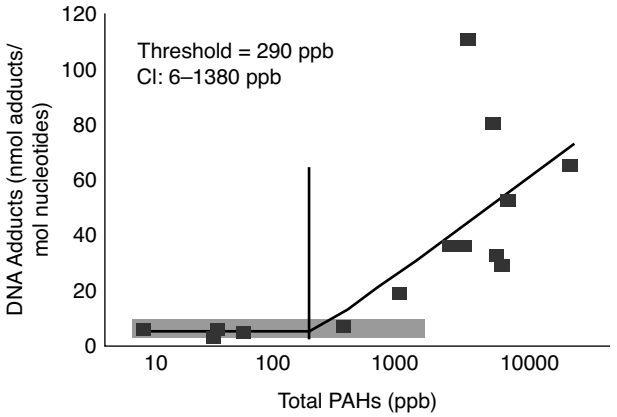

FIGURE 22.18 Hockey-stick regression of PAH–DNA adducts in liver of English sole vs. total polycyclic aromatic hydrocarbons (PAHs) in bottom sediments in ng/g dry wt (ppb) for selected sampling sites in Puget Sound, Washington. Threshold concentration is indicated by arrow. Shaded gray bar represents the 90% confidence interval. (From Johnson, L.L. et al., *Aquat. Conserv.*, 12, 517–538, 2002. With permission.)

Hockey-stick regression, the specific model applied for these analyses, is one of a number of standard dose–response models (Gad and Weil, 1991), and has been used in a variety of epidemiological and toxicological studies (Cox et al., 1989; Gordon and Fogelson, 1993; Hammer et al., 1974). The model consists of two linear segments whose blade-and-handle shape resembles a hockey stick (Yanagimoto and Yamamoto, 1979). The lower segment is assigned a slope of zero to represent a constant low-level background effect. The upper segment is defined as a linear function with a positive slope that represents a dose–response relationship above a threshold that is estimated by the point of intersection of the two segments. An advantage of this approach is that quantitative assessments of uncertainty are provided in the form of confidence intervals for the threshold values.

Many risk analysis models used in epidemiology typically assume that DNA damage and cancer induction are non-threshold phenomenon, and our choice of a threshold model for this exercise is not meant to imply that a true threshold exists in the process of carcinogenesis in English sole. Rather, the model was chosen for pragmatic reasons, to facilitate our identification of exposure levels at which statistically detectable and biologically relevant increases in the endpoints would be expected to occur in wild fish populations. The application of a threshold model is supported by the fact that, for most carcinogens and mutagens, repair processes and compensatory mechanisms exist that can counteract their effects at low levels of exposure, even though one molecule of a carcinogen could theoretically induce an initiated cell, leaving no latitude for a threshold. Based on a similar rationale, the application of a threshold approach for regulating exposure to some carcinogens has been suggested for human health risk management (Butterworth and Bogdanffy, 1999; Gaylor et al., 1999; Lutz, 1998).

We have used this model to relate sediment PAH concentrations to PAH–DNA adducts levels and to prevalences of the four most common toxicopathic hepatic lesion types found in English sole: neoplasms; preneoplastic foci of cellular alteration (FCA), which are thought to be precursors of neoplasms; specific degeneration/necrosis (SDN), a degenerative lesion manifesting cytotoxicity associated with exposure to PAHs; and non-neoplastic proliferative lesions, such as hyperplasia of bile ducts (Figure 22.18 and Figure 22.19) (Johnson et al., 2002). Threshold sediment PAH concentrations for toxicopathic liver lesions in English sole ranged from 54 to 2800 ng/g dry wt. For DNA adducts, the threshold effect estimate was 290 ng/g dry wt., with a 90% confidence interval of 6 to 1380 ng/g dry wt. A threshold in this range is supported by a laboratory study (French et al., 1996) in which exposure to sediments contaminated with 1200 ng/g dry wt. PAH resulted in DNA adduct concentrations in English sole liver of 15 to 20 adducts per mol nucleotides, in comparison with 5 to 6 adducts per mol nucleotides in fish exposed to sediments containing 20 ng/g dry wt. PAH.

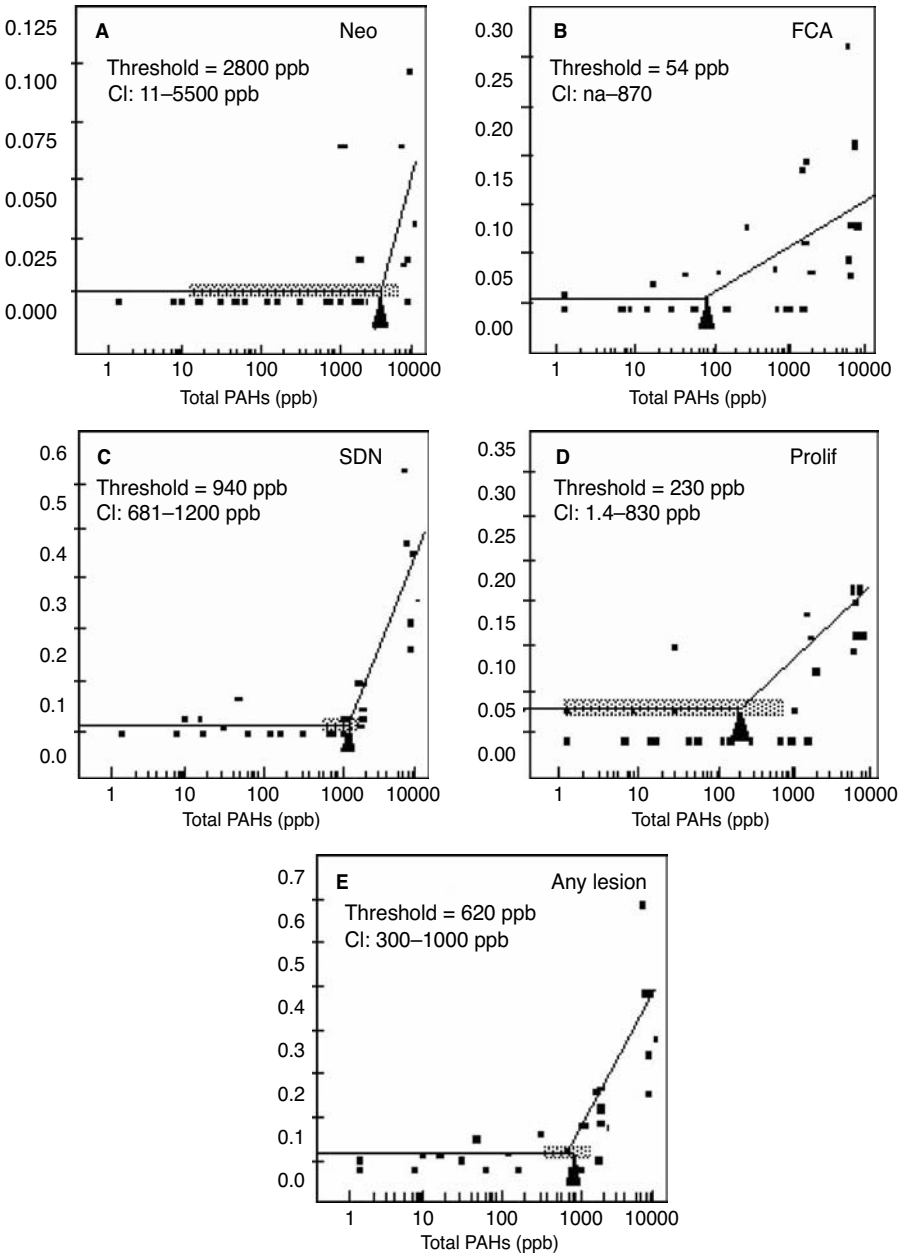

FIGURE 22.19 Hockey-stick regressions of hepatic lesion prevalence in English sole vs. total polycyclic aromatic hydrocarbons (PAHs) in bottom sediment in bottom sediment in ng/g dry wt (ppb) for (A) neoplasms (Neo); (B) foci of cellular alteration (FCA); (C) specific degenerative/necrosis (SDN) lesions; (D) proliferative (Prolif) lesions; and (E) Neo, FCA, or SDN (any lesion). Threshold concentrations are indicated by arrows. Shaded gray bar represents the 90% confidence interval. No lower confidence limit was found for the FCA threshold estimate. $n = 29$. (From Horness, B. et al., *Environ. Toxicol. Chem.*, 17, 162–172, 1998. With permission.)

Although the data are insufficient to statistically determine precise thresholds for other effects in English sole, the available information suggests that impacts on growth and reproduction begin to occur at sediment PAH concentration in a similar range; for example, in Figure 22.20 the prevalences of several

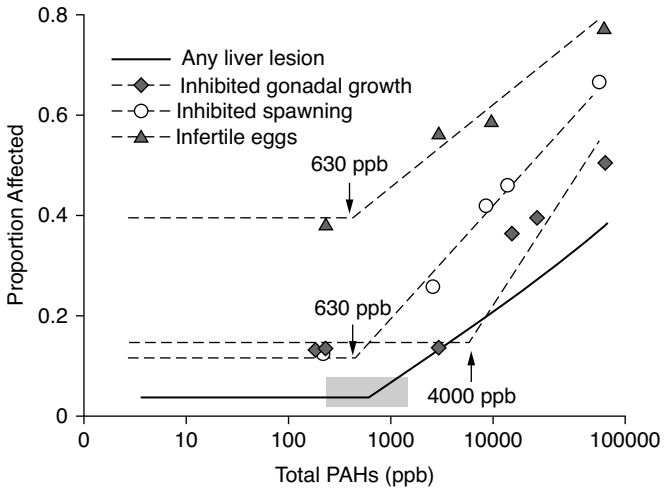

FIGURE 22.20 Measures of reproductive function in female English sole plotted against sediment total PAH concentrations at sites in Puget Sound where sole were collected. For inhibited gonadal growth and inhibited spawning, data points represent the proportion of females at the sampling site that exhibited these conditions. For infertile eggs, the data points represent the average proportion of spawned eggs that were infertile in crosses with sperm from reference males for females from the sampling sites. Dotted lines indicate the hypothetical hockey-stick regression models for reproductive endpoints. The horizontal portion of the curve represents background values for these endpoints, estimated from field data at the reference site with the lowest sediment PAH concentration. Threshold concentrations (indicated by arrows) are the geometric mean of the highest sediment PAH concentration where effect values were at the background level and the lowest concentration where an increased effect level was observed. The upper portion of the curve is the fitted hockey-stick regression model. Estimated threshold and background effect levels were substituted into the model, rather than calculated through regression analysis as for liver lesions. The solid black line represents the fitted hockey-stick regression model and effects threshold estimate for any lesion in English sole (neoplasms, foci of cellular alteration, or specific degenerative necrosis) (Horness et al., 1998). (From Johnson, L.L. et al., *Aquat. Conserv.*, 12, 517–538, 2002. With permission.)

types of reproductive impairment observed in adult female English sole (Casillas et al., 1991; Johnson et al., 1988, 1999) are plotted against sediment PAH concentrations at the sites where fish were collected in Puget Sound. The plots indicate that spawning failure and egg infertility begin to increase above background levels at sediment PAH concentrations between 250 and 1600 ng/g, while the proportion of sole failing to undergo ovarian maturation begins to increase at concentrations between 1600 and 10,000 ng/g. If we estimate the effect thresholds by taking the geometric mean of these two points, this yields an effect threshold of 4000 ng/g for inhibition of ovarian development and a threshold of 630 ng/g for all other reproductive endpoints. Exposure–response relationships for reproductive endpoints at sediment PAH concentrations above the threshold levels were examined by fitting the upper segment of the hockey-stick regression, substituting estimated threshold and background effect levels into the model. The proportion of sole that failed to mature, the proportion that failed to spawn, and the proportion of eggs spawned that were infertile were all significantly correlated with increasing sediment PAH concentration. Although more data would be needed to calculate sediment threshold concentrations and confidence intervals for the reproductive endpoints, the results suggest that these types of reproductive impairment are found at sediment PAH concentrations similar to those associated with the development of hepatic lesions. Currently, we do not have sufficient data on growth rates of field-collected English sole to apply the hockey-stick regression approach to this endpoint; however, preliminary data from laboratory growth studies with English sole (Kubin, 1997; Rice et al., 2000) suggest that significant effects on fish growth are first observed at sediment PAH concentrations between 2000 and 4000 ng/g, consistent with exposure levels associated with liver disease and reproductive impairment.

To estimate the level of effects experienced by English sole exposed to sediments with different sediment PAH concentrations, expected prevalences of liver lesions and reproductive abnormalities were calculated using the regression equations from the hockey-stick analyses for ΣPAH concentrations

TABLE 22.2

Estimated Effect Levels Associated with Increasing Sediment PAH Concentration for Selected Liver Lesions and Indicators of Reproductive Function in English Sole

	Liver Lesions				
PAH (ppb dry wt.)	**Neoplasm (Prevalence)**	**FCA (Prevalence)**	**SDN (Prevalence)**	**Proliferative Lesion (Prevalence)**	**Any Lesion (Prevalence)**
50	0.00	0.01	0.00	0.02	0.00
100	0.00	0.02	0.00	0.02	0.00
1000	0.00	0.06	0.01	0.08	0.09
2000	0.00	0.07	0.12	0.11	0.18
3000	0.01	0.08	0.20	0.13	0.24
5000	0.03	0.09	0.27	0.14	0.31
10000	0.06	0.10	0.38	0.17	0.40
100000	0.16	0.14	0.75	0.26	0.71

	Reproductive Indicators			
PAH (ppb dry wt.)	**Inhibited Gonadal Development (Prevalence)**	**Inhibited Spawning (Prevalence)**	**Infertile Eggs (Proportion of Eggs Spawned)**	**DNA Damage (Adducts per mol Nucleotides)**
50	0.15	0.12	0.38	5
100	0.15	0.12	0.38	5
1000	0.15	0.17	0.42	25
2000	0.15	0.25	0.48	36
3000	0.15	0.30	0.51	43
5000	0.18	0.35	0.55	51
10000	0.27	0.43	0.61	63
100000	0.58	0.69	0.80	100

Note: For all liver lesions, inhibited gonadal development, and inhibited spawning in English sole, the effect level is the proportion of fish estimated to be affected at the indicated sediment PAH concentration; for infertile eggs, the effect level is the proportion of eggs produced by an individual female that are estimated to be unfertile. Effect levels for liver lesions were calculated with hockey-stick regression. For reproductive indicators, effect levels at the sampling sites where PAH concentrations were lowest were used to estimate background effect levels (i.e., effect levels at PAH concentrations below 5000 ppb for inhibited gonadal development and below 1000 ppb for inhibited spawning and infertile eggs).

Source: Adapted from Johnson, L.L. et al., *Aquat. Conserv.*, 12, 517–538, 2002.

ranging from 50 to 100,000 ng/g. As illustrated in Table 22.2, liver lesion prevalences, as well as levels of other detrimental effects in English sole, were generally close to levels characteristic of fish from uncontaminated sites at sediment PAH concentrations below 1000 ng/g. At higher concentrations, the proportion of animals affected and the number of adverse effects observed increases. The degree of increase is modeled by the upper arm of the hockey-stick regression, which can be used to estimate the probability of effects to English sole at various sediment PAH concentrations (Table 22.2). At 5000 ng/g, for example, levels of hepatic DNA adducts would be approximately 10-fold the levels found in fish from uncontaminated reference sites, about 30% of the population is predicted to have some form of toxicopathic liver disease, and the number of fish failing to spawn would increase from about 12% to over 35%. At PAH concentrations of 10,000 ng/g, DNA adducts levels would have increased by 12- to 13-fold, 50% of the sole would be expected to have liver disease, nearly 30% of the females would show inhibition of gonadal growth, and over 40% would show inhibition of spawning. This type of information, in combination with data on contaminant effects on other indigenous species, can be used in estimating the loss of productivity or ecosystem services due to PAH contamination at impacted sites.

Although the current analysis provides guidance on approximate sediment PAH concentrations associated with injury in English sole, some variables that could influence exposure–response relationships are not fully accounted for in the current treatment of the data; for example, the current analysis adjusts

for fish age only in a very basic manner by excluding very young fish and sites where the majority of fish collected were subadults. Also, the thresholds are based on data from urban sites contaminated with a combination of petrogenic and pyrogenic PAHs, and their applicability to estuarine environments where the suite of PAHs present differs substantially from those typically present at industrialized urban sites is not known. Another factor that is not accounted for is the presence of other contaminants along with PAHs at sites where English sole were collected. Although the correlations between PAH exposure and the endpoints we measured in this study are statistically valid and well supported by other scientific evidence, sediments at the sites included in the analysis contain a variety of other compounds that are promoters of carcinogenesis or are reproductive toxicants. These compounds could act either additively or synergistically with PAHs to produce the observed health impacts. Their presence could alter disease prevalence, and they are likely an important factor contributing to variability in response among fish populations at different sampling sites. The potential of interactive effects among co-occurring compounds is an area that warrants additional research because it does introduce uncertainty in the estimation of sediment threshold values.

Overall, these analyses suggest that several important health effects, including selected degenerative liver lesions, spawning inhibition, and reduced egg viability, can be observed in English sole residing at sites where PAH concentrations are approximately 1 to 2 mg/kg and above. Moreover, the proportion of animals affected and the number of adverse effects observed steadily increase as sediment PAH concentrations increase. Additionally, the data suggest that liver lesions could be used as a surrogate to indicate that resident fish are at risk for additional adverse impacts, such as impaired reproduction and growth. With further development of such data, including testing with fish species other than English sole, these relationships could be used to help assess the likely degree of injury to marine resources at various sediment PAH concentrations.

Risk Management

The information generated by our laboratory on the risks posed to marine fishes by exposure to PAHs is being used in environmental management decisions in a variety of settings. One of the most notable has been our involvement in NOAA's Natural Resources Damage Assessment (NRDA) process. Under the Clean Water Act and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), U.S. federal or state officials, acting as trustees for natural resources, can seek compensation from responsible parties for damage caused by releases of toxic materials. Such cases require that a damage assessment be performed to determine what injuries need to be remediated and what compensation is necessary. NOAA, in conjunction with other trustees of marine resources, has pursued damage assessment cases at several sites in Puget Sound, including Elliott Bay in Seattle and Commencement Bay in Tacoma, using PAH-associated health effects on fish as evidence of resource damage. We have also been involved in monitoring efforts to assess the effectiveness of cleanup and restoration activities at Superfund sites in Puget Sound, such as Eagle Harbor (Myers et al., 1995, 2000, 2003, 2005). The following summarizes the salient aspects of a few representative cases.

Puget Sound Damage Assessment Cases

Elliott Bay

In 1990, the United States filed suit against the City of Seattle and Metro (now the King County Department of Metropolitan Services) to recover damages for alleged injuries to natural resources caused by the release of hazardous metals and organic chemicals from the City and Metro combined sewer overflows and storm drains discharging into Elliott Bay and the lower Duwamish River. The parties negotiated a cooperative agreement to work together to restore the natural resources in Elliott Bay and the lower Duwamish River. Under this agreement, the City and Metro provided approximately $24 million in funding, real estate, and in-kind services for sediment remediation, habitat restoration, and source control efforts in Elliott Bay. The information on PAH exposure and associated health effects in

benthic fish in Elliott Bay and the Duwamish, reviewed in the previous section, played an important role in the damage assessment process, because these data established that there had been releases of a series of hazardous substances into Elliott Bay and that public-trust natural resources were injured by the releases. The long-term nature of these studies, the thorough documentation of PAH exposure and biological injury, the consistency of association between PAH exposure and detrimental health effects, and the cause-and-effect relationships established in the laboratory for PAHs and endpoints measured in the field all contributed to the strength and utility of these data.

Commencement Bay

In 1991, the Commencement Bay Natural Resource Trustees (NOAA, U.S. Fish and Wildlife Service, Bureau of Indian Affairs, Washington Department of Ecology, Washington Department of Fish and Wildlife, Washington Department of Natural Resources, and Puyallup and Muckleshoot Indian Tribes) initiated a NRDA process in Commencement Bay, the harbor for Tacoma in southern Puget Sound. A wide range of industrial and commercial concerns, including pulp and lumber mills, marinas, chemical manufacturing plants, and facilities involved in concrete production, oil refining, and food processing, are located along the waterways connected to the Bay. The release of hazardous substances from industries along the waterways and contamination of bottom sediments in the Bay and its waterways posed a potential hazard to marine fish and salmonids that used these areas as rearing and feeding habitat. In fact, because of high contaminant levels in the area, part of Commencement Bay was designated a Superfund site in 1983.

As part of the Commencement Bay Natural Resource Damage Assessment, the Trustees authorized a series of resource injury assessment studies in fish from the Hylebos Waterway, the most heavily contaminated portion of the Commencement Bay site. Fish injury studies were modeled largely on our earlier research at other contaminated sites in Puget Sound, such as the Duwamish Waterway and Eagle Harbor, and included assessments of biological markers of contaminant exposure and injury in juvenile Chinook and chum salmon, toxicopathic conditions in flatfish, and contaminant-induced reproductive dysfunction in English sole (Collier et al., 1998a,b). These investigations demonstrated that flatfish from the Hylebos Waterway exhibited toxicopathic liver disease and reproductive abnormalities similar to those reported from the same species from comparably contaminated sites elsewhere in Puget Sound (Collier et al., 1998a,b; Johnson et al., 1999). They also established exposure to and uptake of PAHs in juvenile Chinook and chum salmon from the Hylebos Waterway (Stehr et al., 2000).

A second set of investigations was conducted to better characterize the uptake and biochemical responses of juvenile Chinook salmon to several classes of contaminants present in the Hylebos Waterway and to determine the effects of these contaminants on growth rate and disease resistance (Arkoosh et al., 2000; Casillas et al., 1998a,b). These studies established that injection of fish with extracts of Hylebos sediment or specific classes of compounds present in Hylebos sediment (e.g., PAHs, PCBs, and hexachlorobutadiene) led to statistically significant reductions in growth and increased mortality in disease challenge experiments. More recently, dietary exposure studies (Bravo, 2005; Meador et al., 2006) have been conducted with juvenile Chinook salmon to better characterize dose–response relationships between contaminants present in the Hylebos and specific injuries and exposure biomarkers in a more realistic exposure regimen.

As the damage assessment has progressed, the Trustees have entered into partial or full settlements of claims with several parties. These settlements provided funds, property, and in-kind services for restoration projects, and restoration activities have been carried out at a number of nearshore and intertidal sites in Commencement Bay (CBNRT, 2001, 2002). In developing these projects, the Trustees had to determine whether and to what extent it was necessary to remediate existing contamination to ensure an acceptable likelihood of success for the proposed restoration project. Washington State sediment management standards (Washington State Department of Ecology, 1995) provided some guidelines for sediment remediation, but these were based primarily on a suite of bioassays with benthic invertebrates, so were not directly applicable to salmonids or bottomfish. Indeed, under the sediment management standards, the sediment quality criteria for PAHs, which are considered to be the concentrations that will result in no adverse acute or chronic health effects in either biological resources or humans, were

set at a level substantially above the sediment PAH thresholds concentrations we associated with the onset of liver disease and other adverse health effects in English sole: ~1 to 5 mg/kg dry wt. total PAH or, for sediment with the 2% organic carbon (OC) content typical of Puget Sound sediments from urban embayments, 50 to 250 mg total PAH per kg OC (Johnson et al., 2002). In contrast, the sediment quality criteria for LAHs* and HAHs** were 370 mg/kg OC and 960 mg/kg OC, respectively. The minimum cleanup levels, which are considered allowable within zones where sediments have been impacted from existing or past discharges, were even higher: 780 and 5300 mg/kg OC (Washington State Department of Ecology, 1995). This discrepancy raised some questions as to whether current standards were adequately protective of marine and estuarine fish.

Concern for the safety of marine resources in Commencement Bay prompted the Trustees to recommended more stringent standards for sediment remediation than those in state regulations. Based on the information generated by studies on sediment PAH concentrations associated with liver disease and reproductive impairment in English sole, they proposed a sediment cleanup goal of 2 mg/kg dry wt. total PAHs (or 100 mg total PAH per kg organic carbon) as the default sediment cleanup goal for active NRDA restoration projects in Commencement Bay. The application of this goal is currently restricted to proposed sites for restoration projects within Commencement Bay, but it poses a challenge to current sediment quality standards for PAHs and sediment quality assessment methods in a wide range of projects and situations. The issue is likely to become increasingly important in the future, with the listing under the Endangered Species Act of Chinook salmon that utilize urban waterways during their migration from fresh to saltwater. Under the Endangered Species Act, adverse health effects to individuals are considered as harm to listed species, as these types of injury could impair the ability of threatened or endangered populations to recover.

Beginning in 2002, the Trustees authorized a set of monitoring studies to determine whether juvenile salmonids and other estuarine fish were utilizing the restored sites and to collect data on contaminant concentrations in fish at the restoration sites (CBNRT, 2001; Olson et al., 2007; Ridolfi and Adolfson, 2003). Target species for contaminant monitoring were juvenile salmon, particularly juvenile fall Chinook, and a resident fish species, Pacific staghorn sculpin. The sculpin were chosen as the target species in lieu of English sole, because the number of sole present at the restoration sites was very limited.

Fish habitat use monitoring (Olson et al., 2007; Ridolfi and Adolfson, 2003) indicated that fish were present at all of the sites, and several supported significant numbers of juvenile salmonids; however, the study also revealed the presence of PAH contamination in fish bile, fish prey, and sediments from all restoration sites (Olson et al., 2007). Concentrations of PAHs in sediments at most of the restoration sites were comparable to levels observed at Commencement Bay sites used as reference areas for the Hylebos Waterway Damage Assessment sediment evaluation studies (EVS, 1996) and were substantially cleaner than sediments in the heavily industrialized sections of the Hylebos Waterway (EVS, 1996). Total PAH concentrations in sediments from a few of the sites, however, were in the range of 8 to 15 mg/kg dry wt., comparable to concentrations measured at some of the more contaminated sites in the Hylebos Waterway as part of the Damage Assessment Study (Collier et al., 1998a,b; EVS, 1996). Similarly, PAH concentrations in salmon bile and stomach contents were lower than those measured in salmon from the Hylebos Waterway during Damage Assessment at most of the restoration sites (Stehr et al., 2000), but concentrations of PAHs in the stomach contents of juvenile salmon from one site were elevated, and bile metabolite levels were above those typically found in salmon from non-urban sites (Johnson et al., 2007). Like juvenile salmon, staghorn sculpin showed exposure to PAHs, based on the presence of polycyclic aromatic compound (PAC) metabolites in bile. Bile metabolite levels in sculpin were lower than those measured in English sole and juvenile salmon collected from the Hylebos Waterway during the Fish Injury Study (Collier et al., 1998a,b; Johnson et al., 1999; Stehr et al., 2000) but still above levels typically found in fish from non-urban sites (Brown et al., 1998).

These studies highlight the importance of prerestoration monitoring for contaminants, especially for projects conducted at urban sites. Although the restoration projects generally provided improved habitat

* LAHs represent the sum of the following compounds: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene.

** HAHs represent the sum of the following compounds: fluoranthene, pyrene, benz(*a*)anthracene, chrysene, total benzofluoranthenes, benzo(*a*)pyrene, indeno(1,2,3,-*c*,*d*)pyrene, dibenzo(*a*,*h*)anthracene, and benzo(*g*,*h*,*i*)perylene.

opportunities for salmon and marine fish within Commencement Bay, in a few cases, enhancements were conducted on habitats that were as contaminated as those that formed the basis of the Hylebos Waterway Damage Assessment case, and existing sediment remediation activities had not yet effectively reduced exposure levels to baseline conditions.

Restoration Monitoring at PAH-Contaminated Sites

The presence of PAH-contaminated sediments in marine environments is widely recognized as a threat to both the ecological and economic viability of coastal regions. A variety of approaches are being used to reduce the environmental risks associated with PAH-contaminated sediments, including source control followed by natural recovery, physical removal or containment of sediments, and *in situ* bioremediation. The effectiveness of these techniques in removing or sequestering contaminants is often carefully monitored, but relatively few studies have been conducted to determine whether or not such treatments reduce contaminant exposure and biological effects in resident fishes. Since 1993, we have had the opportunity to conduct such a study at the Wyckoff/Eagle Harbor Superfund site near Bainbridge Island in Puget Sound.

Eagle Harbor is located near Bainbridge Island in Central Puget Sound, Washington. The 500-acre harbor is heavily used by recreational boaters and for ferry transport to and from Seattle. The mouth of the Harbor was the site of the former Wyckoff Company wood-treatment facility, which operated from 1903 to 1988. As a result of the activities at this plant, marine sediments in the eastern portion of Eagle Harbor are heavily contaminated with creosote-derived PAHs. Studies conducted in the 1980s showed that exposure to PAHs and related compounds were associated with liver cancer, related toxicopathic lesions, and reproductive abnormalities in English sole residing in Eagle Harbor (Johnson et al., 1998; Myers et al., 1998c). Further studies expanded the target species to include starry flounder and rock sole and incorporated additional biological markers of PAH exposure and effect, including hepatic CYP1A expression, biliary FACs, and hepatic DNA adducts, measured by ^{32}P-postlabeling. Hepatic lesion prevalences and other biochemical measures in these three species from Eagle Harbor were among the highest found among Puget Sound sites (Myers et al., 1998b,c; Stein et al., 1992).

Because of its high level of sediment contamination and associated injury to marine life, Eagle Harbor/Wyckoff was designated an EPA Superfund site in 1987. Since that time, several major cleanup projects have been carried out at the site. From 1993 to 1994, a sediment cap of sandy fill material was installed in the East Harbor to contain PAH-contaminated sediments, which was amended and expanded upon in 2000 and 2001. Additionally, the Wyckoff facility has been demolished, and groundwater remediation and soil cleanup efforts are underway. Since completion of the sediment cap installation in the East Harbor in 1994, we have monitored PAH exposure and various effect indicators in English sole and other flatfish species in Eagle Harbor to assess their response following sediment remediation.

Even before the cap was put into place, PAH exposure showed some reduction in Eagle Harbor flatfish in comparison to historical values. This decrease was likely the result of source-control measures enacted at the site between 1988 and 1993. Initially, placement of the cap did not have a clear effect on PAH concentrations in English sole stomach contents or in biliary FAC levels in English sole bile. These data suggested that English sole, because of their preference for fine-grained sediment, were occupying the PAH-contaminated areas peripheral to the cap and not using the remediated habitat. With time, however, PAH exposure levels in English sole from Eagle Harbor have declined significantly. Data collected through 2004 following capping in 1993 showed significant reductions in indicators of PAH exposure (PAH metabolites in bile, PAH–DNA adducts), as well as dramatic and highly significant reductions in prevalences of and relative risks for liver neoplasms and related lesions associated with PAH exposure (Myers et al., 2003, 2005). Stated in terms of estimated relative risk, the risk of lesion occurrence has been reduced by 0.9813 times for each month since the capping process was begun. The decline in risk of lesion occurrence is especially marked from the fourth year since capping was begun (December 1997); since that sampling point, relative risks have been in the vicinity of 0.1 (as compared to 1.0 at the beginning of capping), and age-adjusted relative risks of toxicopathic hepatic lesion occurrence have stayed consistently low (0.05 to 0.36). Overall, this study demonstrates that with a good understanding of cause-and-effect relationships between contaminants and biological health effects and consistent long-term monitoring we can show biological responses in resident organisms to contaminant control and remediation measures.

Conclusion

Field and laboratory data on flatfish in Puget Sound indicate that exposure to PAHs is associated with increases in disease and alterations in growth and reproductive function that could potentially reduce the productivity of fish subpopulations residing at contaminated sites. Studies also suggest that even short-term exposure to PAHs may be associated with reduced growth and altered immune function in anadromous fish species that utilize contaminated estuaries in Puget Sound. Similar biological effects have also been observed in fish in urban coastal waters throughout Europe and the United States, suggesting that marine pollution and other forms of habitat degradation may be a widespread threat to fish species, particularly those that rely on nearshore coastal areas for nursery and feeding grounds. The studies with English sole substantiate the value of a multidisciplinary approach that combines field and laboratory experiments with quantitative modeling techniques in evaluating the ecological risk to fish stocks of exposure to chemical pollution. The application of such an approach to other fish species should yield much interesting information on species differences in sensitivity and the factors that predispose certain species or stocks to pollution-related impairment, and they improve our ability to protect these stocks from contaminant-related health injury. Although the effects of PAHs on the health of Puget Sound fish have been recognized for over two decades, the problem has not diminished, except in a few urban areas (Seattle Waterfront in Elliott Bay and Thea Foss Waterway in Commencement Bay) where recent data from the Puget Sound Ambient Monitoring Program indicate significant decreases in toxicopathic liver lesion risk since 1998 (PSAT, 2004). Because of changes in demography and urban growth in the Puget Sound Areas, PAHs still pose significant ecological risks to resident bottomfish.

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23

Effects of the Exxon Valdez Oil Spill on Pacific Herring in Prince William Sound, Alaska

Gary D. Marty

CONTENTS

Background*

On March 24, 1989, the *Exxon Valdez* ran aground on Bligh Reef in the northeastern part of Prince William Sound (PWS), spilling about 40×10^6 L of crude oil. This was the largest crude oil spill in U.S. waters, and it occurred in what otherwise was a relatively pristine, highly productive, but highly sensitive ecosystem. After three days of calm weather and smooth seas, strong northeasterly winds arose and dispersed the oil beyond any hope of containment. The spilled oil, soon in the form of thin sheens and thick mousse, continued to spread to the southwest (Short and Harris, 1996). Control was difficult due to variable weather conditions and tide cycles that ranged up to 6 m. The oil came ashore along an approximately 750-km trajectory from PWS to the southern Kodiak Archipelago and Alaska Peninsula. The distribution and depth of the oil along the shoreline were discontinuous and variable, both on the surface and within the beach substrate. Estimates vary, but about 20% of the spilled oil evaporated, 40% was deposited on beaches within PWS, and about 10% entered the Gulf of Alaska (Spies et al., 1996). By fall 1992, only about 2% of the spilled oil remained on the beaches (Spies et al., 1996). Field surveys conducted in the summer of 1989 identified 720 km of oiled shoreline within PWS, over 300 km of which was classified as heavily oiled.

Work after the spill was divided into three phases: *response*, *damage assessment*, and *restoration*. Management of response was an effort coordinated by the U.S. Coast Guard, the Alaska Department of Environmental Conservation, and Exxon, but other federal and state agencies and local communities played key roles. Both damage assessment and restoration activities were managed by the State of Alaska and three federal agencies acting together as Natural Resource Trustees as provided by the Comprehensive

* Ecosystem-level impacts of the spill are reviewed elsewhere (Peterson et al., 2003). The background information in this section is adapted with permission (public domain) from the website of the *Exxon Valdez* Oil Spill Restoration Office (http://www.evostc.state.ak.us/).

Environmental Response, Compensation, and Liability Act (CERCLA). This act also provided the statutory basis for compensating the public for injuries to natural resources resulting from spills of hazardous substances. The organizational structure that was established for the restoration phase was guided by the Memorandum of Agreement and Consent Decree, which was filed in the U.S. District Court for the District of Alaska in civil action A91-081 (*United States v. State of Alaska*) and approved and entered by U.S. District Judge H. Russell Holland on August 28, 1991. Under this agreement, three federal and three state trustees were made responsible for administering the restoration fund and restoring injured resources and services.

The response effort involved removing unspilled cargo, vessel salvage, booming of sensitive areas, beach surveys and assessments, overflights to track the floating oil, skimming of floating oil, cleanup of oiled beaches, wildlife rescue, waste management, logistics support, and public relations. Major cleanup operations were conducted during the spring and summer of 1989 to 1992, at a cost of more than $2 billion. Thousands of workers were involved in cleanup and logistics support operations that required hundreds of vessels, aircraft, and a substantial land-based infrastructure. Techniques used to remove or clean oil included burning, chemical dispersants, high-pressure/hot-water washing, cold-water washing, fertilizer-enhanced bioremediation, and manual and mechanical removal of oil and oil-laden sediments. Cleanup operations on the beaches during the first four summers led to the recovery and disposal of approximately 31,000 tons of solid oily wastes, which were estimated to account for 5 to 8% of the original spill volume. About 90% of the oil in surface beach sediments (<25 cm depth) was removed by natural processes (storm erosion and biodegradation) during the winter of 1989/1990, whereas only about 40% of the deeper oil was removed. By 1992, the combination of natural processes and cleanup activities had eliminated nearly all of the surface oil, although small amounts persisted along many shoreline segments in PWS.

During the first summer after the spill (1990), Natural Resource Damage Assessment studies were begun to assess the injury inflicted by the spill. Many of these studies were carried out through 1992. It was the largest and most extensive damage assessment program in U.S. history, with more than $100 million devoted to 164 separate and related studies. Studies were evaluated from five perspectives: (1) immediate injury, (2) long-term alteration of populations, (3) sublethal or latent effects, (4) ecosystem-wide effects, and (5) habitat degradation. These studies formed the scientific basis from which the United States and the State of Alaska would conduct their litigation against Exxon. After the U.S. District Court approved an agreement that settled the claims of the governments for criminal violations and recovery of civil damages, these damage assessment studies were used to guide restoration. This body of work was designed to provide information on the nature and extent of injury and the status of recovery for an injured resource or service.

The oil spill caused injury at virtually all trophic or organizational levels; however, the extent and degree of injury were uneven across the oiled landscape. Some species were only slightly affected (e.g., brown bear and Sitka blacktail deer), whereas others (e.g., the common murre and the sea otter) suffered population-level injuries, with possible long-term consequences. The complex issues of determining injury and recovery from the spill are highly controversial and are still being argued in the courts, at scientific meetings, and in the literature (Brannon et al., 2001; Carls et al., 2002). The spill severely impaired southcentral Alaska's fisheries, the foundation for most of the region's small communities, and had severe social and psychological consequences for the area's human population. The *Exxon Valdez* Oil Spill Trustee Council decided that natural resource injuries from exposure to the spill or from the cleanup would include: (1) *mortality*—death caused immediately or after a period of time by contact with oil, cleanup activities, reductions in critical food sources caused by the spill, or other causes; (2) *sublethal effects*—injuries that affect the health and physical condition of organisms (including eggs and larvae) but do not result in the death of juvenile or adult organisms; and (3) *degradation of habitat*—alteration or contamination of flora, fauna, and the physical components of the habitat.

The historical crux of the oil spill, with regard to restoration, was the agreements between the State of Alaska and the United States with Exxon, approved by the U.S. District Court on October 8, 1991, on both criminal charges and civil damage claims. This decision eliminated the need for expending millions of dollars and years of time in litigation with Exxon and instead provided money and human resources for restoration work. In the civil settlement, Exxon agreed to pay the State of Alaska and the United States $900 million over a 10-year period. According to the terms of the settlement, restoration

funds had to be used "for the purposes of restoring, replacing, enhancing, or acquiring the equivalent of natural resources injured as a result of the Oil Spill and reduced or lost services provided by such resources." In addition to acquisition of sensitive habitat, restoration involves two types of research projects: (1) recovery monitoring projects that track the rate and degree of recovery of resources and services injured by the spill and that also may determine when recovery has occurred or detect reversals or problems with recovery; and (2) research projects that provide information required to restore an injured resource or service or information about ecosystem relationships. Detailed studies were undertaken on a number of different species. The objective of this case report is to describe the response, damage assessment, and early restoration studies with Pacific herring (*Clupea pallasi*).

The most recent detailed survey of lingering oil was conducted in the intertidal zone of PWS in the summer of 2001 by the U.S. National Oceanic and Atmospheric Administration (NOAA) Auke Bay Laboratory (Short et al., 2004). Approximately 11.3 ha of shoreline in PWS were still contaminated with oil. Oil contaminated 86% of the 91 sites assessed and is estimated to have the linear equivalent of 5.8 km of contaminated shoreline.

Life History and Background

Pacific herring are members of the family Clupeidae, which is abundant throughout the North Pacific. Pacific herring in PWS first spawn when 3 to 5 years old. Although they spawn annually in April, abundant year classes recruit into the fishery only sporadically (about every 4 years). Pacific herring in PWS weigh up to 300 g and can live to be 15 years old, but few fish survive longer than 10 years. They feed primarily on zooplankton, which is most abundant from May through October. Because Pacific herring tissues have high lipid content, they are a very important high-energy food source for marine mammals, birds, and other fish. Commercial fisheries harvest Pacific herring for roe (eggs) and for use as bait, with a value of about $8 million/year in the 1980s. When the spill occurred in 1989, Pacific herring populations in PWS were the highest in 20 years of reliable estimates (>1 billion adults). All life stages were susceptible to damage from the spill. Spawning occurs in shallow bays, and eggs are deposited and stick to kelp and rocks. Eggs hatch about 21 days after spawning. Resultant larvae are planktonic (i.e., they drift with the currents) until they are large enough to maintain position against the currents (about 2 months after hatch). Movements of juveniles were mostly unknown when the spill occurred, but we have since learned that they congregate in small schools scattered in bays throughout PWS year-round (Norcross et al., 2001). By comparison, adults aggregate in only a few areas from fall until early spring. One of the largest fall-to-spring aggregations of adults during the 1990s was in the bays of northern Montague Island. When adults were abundant, prespawning aggregates of fish in this area were as large as several kilometers long and 2 to 3 km wide. Postspawning fish disperse throughout the Sound during the spring and summer feeding season. The major concern for spill-related effects on adults was that they were in spawning aggregations when the oil spill occurred. Other reviews have provided detailed maps of the spill trajectory and described some of the impact of the oil spill on Pacific herring (Brown et al., 1996; Carls et al., 2002; Kocan and Hose, 1997). The objective of this case report is to demonstrate general principals of damage assessment after an oil spill using Pacific herring and the *Exxon Valdez* oil spill as the model.

Post-Spill Damage Assessment

Three types of evidence must be documented for complete damage assessment:

- *Potential for exposure*—Quantify hydrocarbons in water and mussels.
- *Actual exposure*—Quantify hydrocarbons or their metabolites in tissues; this could also include determination of cytochrome P450 levels (e.g., CYP1A).
- *Effect*—Describe and quantify alterations in behavior, reproduction, growth, tissue morphology (e.g., gross or microscopic lesions), or mortality.

Although many of the desired components were studied, some parts of the assessment puzzle are incomplete. When data were incomplete, findings were compared with results of concurrent laboratory studies or with previously published results.

Measurements of Potential for Exposure

The potential for exposure was documented by measuring hydrocarbon concentrations in water. Several agencies and individuals recorded visual observations of surface oiling, but these observations often varied. There seemed to be less controversy over hydrocarbon concentrations measured in the water column. Exxon-funded scientists reported that polynuclear aromatic hydrocarbons (PAHs) in surface water (depth up to 5 cm) from oiled sites averaged 0.9 µg/L in late April and early May 1989 (Neff and Stubblefield, 1995). Similarly, Trustee Council-funded scientists reported that PAH concentrations at a depth of 1 m were 0.5 to 1.0 µg/L in mid-April, but at those depths PAHs were detectable only near heavily oiled beaches by early May (Short and Harris, 1996). Both studies reported no significant PAH residues in the water column by 1991. Fish tissues—including eggs—rapidly metabolize petroleum hydrocarbons (Collier and Varanasi, 1991; Thomas et al., 1997); therefore, analysis of these samples usually does not provide an accurate assessment of cumulative exposure over a period of several days. Exposure to sessile life stages such as eggs can be better estimated by determining hydrocarbon concentrations in mussels sampled from the same areas as eggs. In PWS, mussels were selected as a good model for previous hydrocarbon exposure (Hose et al., 1996) because they do not readily metabolize petroleum hydrocarbons.

The primary controversy over the measured hydrocarbon concentrations in water was the potential significance of the findings. The water-soluble fraction* of crude oil is toxic to Pacific herring embryonic development at 0.1 to 1.0 mg/L (Cameron and Smith 1980; Kocan et al., 1996a; Smith and Cameron, 1979). Based in part on these reports, Exxon-funded scientists concluded that "it is likely that the safety factor (ratio of effects concentration to environmental concentration) for marine organisms in PWS following the spill was in excess of 100 and possibly as high as 10,000 during April and May 1989" (Neff and Stubblefield, 1995). In contrast, evidence provided by Trustee Council-funded scientists from field and laboratory studies supported the conclusion that hydrocarbon levels in PWS were indeed significant (see the The Effects of Exposure section, below) (Carls et al., 1999; Marty et al., 1997). The reason for this difference is that earlier studies determined toxicity based on the concentration of the water-soluble fraction of crude oil, but the PAH component of crude oil is more toxic on a molar basis (Lee and Page, 1997).

Measurements of Actual Exposure

Actual exposure was determined by measuring hydrocarbons or their metabolites in fish tissues. Exxon-funded scientists identified hydrocarbons in eggs from highly contaminated sites (Pearson et al., 1995). Trustee Council-funded scientists did not measure hydrocarbon concentrations in eggs; instead, they determined the hydrocarbon concentrations in nearby mussels. Actual hydrocarbon exposure to Pacific herring juveniles was not reported, but exposure was reported in adults. Tissue PAH concentrations in 1989 were higher in adults from oiled sites than in adults from reference sites, but site-related differences were no longer significant in 1990 (Marty et al., 1999). Corroborative evidence that Pacific herring were exposed to *Exxon Valdez* oil includes contaminated or possibly contaminated bile in two of two herring examined in 1989 (Haynes et al., 1995).

The Effects of Exposure

The effects of the oil on Pacific herring were documented in several studies. In 1989, developing eggs were removed from reference sites and from sites that were thought to have been exposed to oil in PWS; they were incubated in the laboratory and examined for abnormalities less than 24 hours after

* *Water-soluble fraction vs. polynuclear aromatic hydrocarbons:* Primary components of the water-soluble fraction of crude oil include low-molecular-weight compounds such as mono- and bicyclic hydrocarbons. Polynuclear aromatic hydrocarbons have relatively higher molecular weights and include naphthalenes, phenanthrenes, dibenzothiophenes, fluorenes, and chrysenes.

hatch. When Exxon-funded scientists examined the resultant larvae for abnormalities that could be detected using a dissecting microscope (Pearson et al., 1995), they found no relation between lesions and egg PAH levels and concluded that 4% of miles of herring spawn were on oiled beaches. By comparison, Trustee Council-funded scientists examined resultant larvae using gross examination, cytogenetic analysis (e.g., morphology of mitotic figures in fin epidermal cells), and histopathology (Hose et al., 1996). They found that significant morphological and cytogenetic abnormalities were significantly correlated with PAH concentrations in mussels collected from the same area as eggs, and these results were confirmed with laboratory study (Carls et al., 1999; Kocan et al., 1996a). Trustee Council-funded scientists concluded that more than 25% of the egg biomass was deposited within the oil trajectory (Brown et al., 1996; Carls et al., 2002). Eggs deposited in 1990 and beyond had no evidence of spill-related effects.

Two studies of Pacific herring larvae in 1989 revealed that the spill had several significant effects. In the first study, staggered plankton trawls in oiled and reference sites in nearshore areas of PWS through May and June of 1989 provided evidence that larval mortality in oiled areas was two times greater than in reference areas (McGurk and Brown, 1996). Microscopic analysis of a subset of the trawled larvae revealed that larvae from oiled sites were shorter, had ingested less food, and had evidence of slower growth (Marty et al., 1997); the prevalence of fish with cytogenetic damage in this subset was also greater in fish from oiled areas (oiled, 56 to 84% had damage; reference, 32 to 40% had damage). Prevalence of ascites (i.e., accumulation of fluid in the coelomic/peritoneal cavity) was greater in fish from oiled sites (16%) than in fish from reference sites (1%) (Marty et al., 1997). As corroborative evidence that the spill was responsible for these changes, laboratory study demonstrated that 50% of Pacific herring developed ascites after continuous exposure to 0.8-µg/L PAH during embryonic and early larval development (Carls et al., 1999). Ascites and pericardial edema (i.e., accumulation of fluid around the heart) are probably irreversible lethal lesions in most affected fish.

In a second field study, plankton trawls were done in offshore areas of PWS during May, June, and July of 1989 (Norcross et al., 1996). Currents in offshore areas had mixed the planktonic larvae such that the potential oiling history of larvae captured offshore could not be determined; however, many larvae exhibited symptoms associated with oil exposure in laboratory experiments and other oil spills. Effects included morphological malformations, genetic damage, and small size. Growth between May and June 1989 was the lowest ever reported for field-caught larval Pacific herring throughout their distribution (Norcross et al., 1996). Some of the offshore trawls were repeated 6 years later; in May 1995, jaw and cytogenetic development in Pacific herring larvae were normal and significantly different from larvae in 1989 (Norcross et al., 1996). Despite very large spawn deposition in 1989, that year's class turned out to be one of the smallest to be recruited into the fishery; by comparison, the spawning biomass of the 1995 year class was less than 25% of the 1989 year class, but recruitment of the 1995 year class into the fishery was above average. Although it is tempting to conclude that the oil spill was responsible for poor recruitment of the 1989 year class, recruitment of the 1989 year class was also poor in Sitka Sound (G.D. Marty and Alaska Department of Fish and Game, unpublished data), which was used as a reference site for the PWS Pacific herring population (Hose et al., 1996; Pearson et al., 1995). Thus, oceanographic variables probably were more significant in limiting recruitment of the 1989 year class in PWS than was the oil spill (Marty et al., 1997).

Effects of the oil spill on Pacific herring juveniles were not reported, but significant effects were found among adults. Unfortunately, small sample size in 1989 prevented the extrapolation of these findings to the population level. In 1989, 20% of 20 adults sampled from oiled sites had hepatic necrosis (liver cell death, determined using histopathology), but none of the fish from reference sites had hepatic necrosis (Marty et al., 1999). Also, fish sampled from oiled sites had fewer herring worms (Anisakidae) in their visceral cavity than did fish from reference sites, and laboratory study confirmed that parasite numbers in the visceral cavity significantly decreased after oil exposure (Moles et al., 1993). Hepatic necrosis was initially thought to be a direct toxic effect of oil; however, subsequent laboratory study revealed that hepatic necrosis in Pacific herring can be caused by expression of viral hemorrhagic septicemia virus (Kocan et al., 1997), and expression of viral hemorrhagic septicemia virus can be induced in adults by dose-dependent exposure to weathered crude oil (Carls et al., 1998). The link between oil exposure and viral expression is probably a nonspecific complication following stress, and fish are most susceptible

to stress at the end of the winter as they come into spawning condition. As evidence, viral hemorrhagic septicemia virus has been isolated several times from adult Pacific herring in Washington, British Columbia, and Alaska, and these areas were not exposed to oil spills (Meyers and Winton, 1995). In a detailed study of disease in the Pacific herring population of PWS from 1994 through 2002, both hepatic necrosis and viral hemorrhagic septicemia virus were common during some years in spring samples but not fall samples, and these findings were considered to be independent of any direct effects from *Exxon Valdez* oil (Marty et al., 2003).

Among adult Pacific herring examined in 1990, fish from oiled sites had significantly greater histopathology scores for pigmented macrophage aggregates. Pigmented macrophage aggregates are irregular spherical structures that are a normal component of organs such as the liver, kidney, and spleen. Lipofuscin and iron are the most common non-melanin pigments, and three main functions have been identified (Wolke, 1992): (1) immunity; (2) storage, destruction, or detoxification of substances produced inside the fish (tissue components) or outside the fish (e.g., toxins in the water); and (3) iron recycling. Pigmented macrophage aggregates are rare in Pacific herring before they first spawn, but they increase in size and number as fish age. In samples from 1990, the age of fish from the oiled site (mean, 5.6 years) was greater than the age of fish from the reference site (mean, 2.3 years); therefore, differences in pigmented macrophage aggregates were more likely a result of age differences in the sample than due to oil exposure (Marty et al., 1999; Wolke, 1992). In 1991, no oil-related effects were detected among adults. In 1992, fish from the 1988 year class that spawned in previously oiled areas had less reproductive success than fish spawning in reference areas; reproductive success was significantly related to microscopic lesions but not to oil history of the sites (Kocan et al., 1996b). In the fall of 1992, a record Pacific herring biomass was predicted for the roe harvest in April 1993. The Pacific herring population of PWS was considered fully recovered from effects of the spill, and all Pacific herring study was terminated.

In 1993, the population crashed. More than 700 million adult Pacific herring, 70 to 80% of the population, disappeared between the fall of 1992 and the spring of 1993. Between 15 and 43% of the fish that returned to spawn in 1993 had ulcers and hemorrhage of the skin and fins (Meyers et al., 1994); as soon as the magnitude of the problem was realized, all Pacific herring fisheries were closed. The cause was probably not directly related to oil exposure but might have been a secondary response to management decisions that increased the population density after the spill. A detailed analysis of potential causes of the population crash and a potential link to the oil spill can be found elsewhere (Carls et al., 2002; Pearson et al., 1999). Viral hemorrhagic septicemia virus was isolated from fish sampled in 1993 (Meyers et al., 1994), and the virus probably contributed significantly to the death of many of the fish. After another year of population decline, detailed study in 1994 revealed no evidence that fish exposed to the oil spill in 1989 as larvae or juveniles had increased disease prevalence (Elston et al., 1997; Marty et al., 1998). The cause of the population crash likely was a complex interaction among the fish, environment, and the diseases or pathogens to which the fish were exposed. Comprehensive semiannual disease study of the Pacific herring population of PWS through 2002 clearly demonstrated a significant link between disease prevalence and changes in population biomass (Marty et al., 2003).

Summary

Oil spilled from the *Exxon Valdez* caused significant damage to Pacific herring larvae and adults in 1989. Decreased concentrations of oil in the water column by 1990 were correlated with an absence of significant effects in Pacific herring in 1990 and beyond. Severe population decline occurred just 4 years after the spill, but direct links to the spill were not detected. Because Pacific herring commonly are infected with a potentially deadly virus (viral hemorrhagic septicemia virus), and stress results in virus outbreaks, Pacific herring are unusually sensitive to spilled oil or other abnormal stressors during physiologically demanding periods of their life cycle such as spring spawning. As a result, oil spills that overlap with Pacific herring spawning are likely to cause greater population damage than oil spills that occur at other times of the year.

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24

Case Study: Pulp and Paper Mill Impacts

Monique G. Dubé, Kelly R. Munkittrick, and L. Mark Hewitt

CONTENTS

Introduction

Examination of the environmental effects of effluent discharges from pulp and paper mills provides an interesting case study in fish toxicology (Figure 24.1). The effects of pulp mill effluents (PMEs) on aquatic environments have been examined for over 40 years, and extensive studies have been conducted since the mid-1980s. During this period, environmental effects have been observed, regulations have been implemented, and the industry has responded to these regulations, resulting in significant reductions in environmental effects. In the 1970s, many effluents received no secondary treatment, and environmental concerns were associated with oxygen demand, suspended solids, and nutrient and organic loading (Owens, 1991). These concerns shifted in the mid-1980s when a series of Swedish field studies documented that PMEs induced toxic responses in fish at very low concentrations (Södergren, 1989). Over the next few years, supporting data were obtained in Canada (Hodson et al., 1992; McMaster et al., 1991; Munkittrick et al., 1991, 1992a,b; Servos et al., 1992), the United States (Adams et al., 1992), and New Zealand (Sharples and Evans, 1996; Sharples et al., 1994) confirming that effluent from some

FIGURE 24.1 Photographs of pulp mills, pulp mill effluent discharge, field sampling, and examples of target sentinel species: creek chub (*Semotilus atromaculatus*) and slimy sculpin (*Cottus cognatus*).

discharges was capable of affecting fish at concentrations lower than previously suspected. It has since become clear that some PMEs impact the reproductive health of fish, yet the factors determining which mills will cause effects are unknown (Environment Canada, 2003; Lowell et al., 2004; Sandström, 1996).

Various countries have used different strategies for regulating PME discharges (Folke, 1996). Regulations can focus on the technology used, the treatment required, chemical concentrations in the effluent, the biological toxicity, or maintenance of a specified level of receiving environment quality. In Canada, for example, chlorinated dioxins in some effluents and evidence of continued environmental issues at modernized mills resulted in amendments in 1992 to the Canadian Pulp and Paper Effluent Regulation under the federal Fisheries Act (Environment Canada, 1992a, 1997a). Under the revised regulation, final effluents could not be acutely lethal to fish, and limits were set for the discharge of organic compounds (measured as biochemical oxygen demand) and chlorinated dioxins. Furthermore, amendments included an Environmental Effects Monitoring (EEM) requirement, where each mill is required to monitor receiving waters and effluent quality on a cyclical basis (Environment Canada, 1998). To meet these more stringent regulations, the Canadian pulp and paper industry made a significant investment to modify the processes used to whiten pulp (i.e., substituted elemental chlorine with chlorine dioxide in the bleaching process), and most mills installed biological effluent treatment systems (Folke, 1996; Karels and Oikari, 2000; Kovacs et al., 2003; Munkittrick et al., 2000a). These process changes virtually eliminated acute lethality of PMEs to fish and the discharge of persistent chlorinated organic compounds such as dioxins. Similar regulatory changes and mill process improvements have occurred internationally (Folke, 1996).

Improvements in effluent quality have changed the types of responses measured in receiving environments exposed to PMEs. If effects are observed, they are sublethal in nature. In Canada to date, the dominant response pattern of freshwater benthos is one of nutrient enrichment (Environment Canada, 2003; Lowell et al., 2004). The dominant fish response patterns include nutrient enrichment (increased condition, liver and gonad size) and altered energy allocation (increased condition and liver size, decreased gonad size). These results are consistent with those reported for some mills in the United States (Adams et al., 1992; Sepúlveda et al., 2003) and Scandinavia (Karels et al., 1999; Larsson and Forlin, 2002).

The objective of this chapter is to present the history of pulp and paper effects assessment on fish as a case study in fish toxicology. Overviews are provided of the pulp and paper process, historical impacts of effluents, regulatory changes, industry improvements, and methods used for assessing effects. Current gaps in knowledge and active areas of research and development are also discussed.

The Pulp and Paper Industry

Paper and paper products are essential to people living in modernized societies. As a result, the pulp and paper industry plays a vital role in the global economy. Approximately 15% of the world's paper mills are in North America, and these mills produce 36% of its paper (Smook, 1994). Canada alone is one of the largest producers and exporters of pulp and paper; it supplies 34% of the world's wood pulp exports and more than 50% of its newsprint, and the forest sector in general employs over 1 million people (FPAC, 2002). To understand how PMEs affect fish, it is important to grasp the fundamentals of pulp production. Each pulp mill is unique, employing different techniques to remove the fiber from trees, including thermo-mechanical processes and chemical processes. Each mill has a unique combination of equipment and processing options, tailored to meet the specific needs of the customers for their pulp and the nature of the product (e.g., newsprint, cardboard boxes, tissue paper, photographic paper, writing paper). Some mills have an integrated paper facility, while others produce only pulp. Furthermore, many mills have multiple lines, including thermomechanical and chemical processes, and some mills alternate processes, fiber sources, and bleaching sequences (e.g., chlorine, chlorine dioxide, hydrogen peroxide), depending on the needs of a specific customer. In many cases, a paper mill may buy their pulp from other sources and have no on-site pulping facilities.

The basic principle of pulping is to convert wood chips into fibrous raw material called pulp, which is a suspension of wood cellulose fibers in solution. Wood consists of the fibrous carbohydrates cellulose (45 to 50%) and hemicellulose (25% for softwoods), lignin (25 to 35% for softwoods), and compounds easily extracted from the wood (2 to 8%), such as terpenes, resin and fatty acids, plant sterols, and phenols (Biermann, 1996; LaFleur, 1996; NCASI, 1989; Smook, 1994). Cellulose is the backbone component of wood fiber and is the most important component for pulp production. Hemicelluloses are the filler of the cellulose fiber and are more soluble and labile than cellulose, resulting in greater susceptibility to degradation in chemical pulping. Lignin is a high-molecular-weight polymer that binds or cements the cellulose fibers together in a matrix (Kringstad and Lindström, 1984). The objective of pulping is to separate and recover the cellulose fibers from the lignin and other wood constituents with maximum fiber yield and minimum fiber degradation (LaFleur, 1996).

The most common pulping process is the kraft (or sulfate) process that was patented by C. F. Dahl in 1854 as a modification of the soda process (Smook, 1994). In the kraft process, wood chips are digested, or cooked, at high temperature (160 to 180°C) and pressure (800 kPa) with white liquor, which is a mixture of hot caustic soda (NaOH) and sodium sulfide (Na_2S) (Biermann, 1996) (Figure 24.2). The lignin and wood extractives are solubilized in the cooking chemicals, leaving the less soluble cellulose fibers as pulp (Kringstad and Lindström, 1984; McCubbin and Folke, 1992; McLeay and Associates, 1987). After digestion, the pulp is washed, screened, and cleaned in the brownstock washing area, and the pulp fiber is separated from the residual weak black liquor. Weak black liquor is a complex mixture containing waste lignin, cooking chemicals, and wood extractives (Biermann, 1996). Following brownstock washing, the pulp may be sent to a bleach plant, where residual lignin is removed and the pulp is bleached to achieve a desired brightness. At some mills, pulp is sent through an oxygen delignification stage to remove additional lignin prior to bleaching (Figure 24.2). This reduces the amount of lignin entering the bleach plant and decreases the volume of bleaching chemicals required (NCASI, 1989).

In the bleaching process at bleached kraft pulp mills (BKPMs), pulp is treated in multiple, alternating stages with various bleaching chemicals containing chlorine or oxygen followed by extractions with alkali (Biermann, 1996). Due to environmental concerns, bleaching technologies have changed throughout the years. Elemental chlorine was the dominant bleaching agent in the 1970s and 1980s and was largely replaced by chlorine dioxide (elemental chlorine-free [ECF] bleaching) in the 1990s (Folke, 1996; Johnson et al., 2003). Complete elimination of bleaching using chlorine-based compounds (totally chlorine free [TCF]) is also an emerging technology.

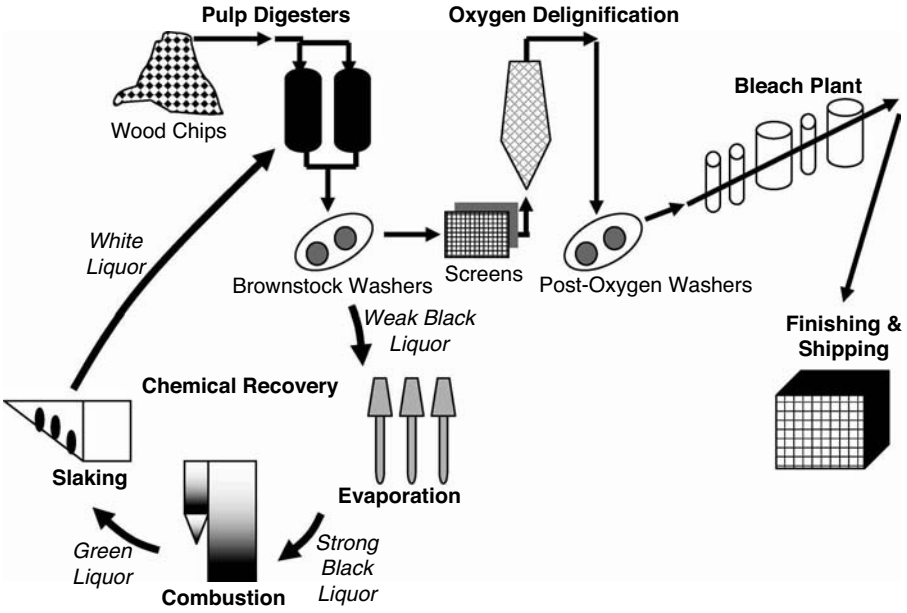

FIGURE 24.2 Kraft pulp and paper process schematic showing digestion of chips to produce pulp, bleaching of the pulp, and the chemical recovery cycle.

To recover the cooking chemicals used in pulp digestion, weak black liquor is sent through the chemical recovery phase of the mill operations (Figure 24.2). Modern chemical recovery systems are designed to recover 96 to 99.5% of the spent cooking chemicals (McCubbin and Folke, 1992). This phase of operations transforms weak black liquor to strong black liquor through a series of evaporators and combusts it in a recovery furnace to form inorganic smelt. The smelt is dissolved in water to form green liquor, which is causticized with lime to regenerate white cooking liquor (McLeay and Associates, 1987; Smook, 1994).

In addition to the production of pulp, a mill produces large volumes of waste effluent. A typical BKPM (700 tonne/day capacity) can discharge between 90 and 130 million liters per day of effluent into surface waters (lakes, rivers, estuaries, oceans) (Walden, 1976). In Canada, effluent concentrations in receiving waters range between 0.2% (100 m from the outfall on the Abitibi River, Ontario) and 30% (100 m from the outfall on Riviere du Nord, Quebec) (Environment Canada, 2003). Effluent concentrations vary from mill to mill, depending on the amount of water used per tonne of pulp, the size of the receiving environment, and fluctuations in receiver flow on a seasonal and annual basis. Significant efforts have been made by the pulping industry to reduce freshwater use and, hence, the discharge of effluent. In Canada, pulp and paper mills have reduced their water consumption per tonne of output by 30% since 1990 (FPAC, 2002). Many mills now recycle process streams within the mill (e.g., using condensates from the chemical recovery process for brownstock washing) and use countercurrent washing techniques to reduce water use (LaFleur and Barton, 2003). One Canadian mill has achieved zero discharge and does not release effluent. The final effluent discharged from a BKPM is a complex chemical mixture consisting of several effluents produced at different stages within the process. Effluent produced in the debarking of trees, brownstock washing wastewaters, bleach plant filtrates, black liquor and chemical spills, and condensed vapors that are produced during weak black liquor evaporation (i.e., condensates) are examples of process effluents that can be discharged into the main chemical sewer (LaFleur, 1996; Smook, 1994). Each process effluent has a unique chemical composition and can interact with other effluents, resulting in a complex final mixture. In addition, the quality of effluent will vary with the species of tree used as the fiber source, whether the trees are debarked by wet or dry processes, and whether the fiber source is pulp wood, wood chips, or recycled fibers. As a result, the effluent quality is not static, and a generalization among mills is seldom possible.

FIGURE 24.3 Photographs of aerated stabilization basins used to treat pulp and paper effluents prior to discharge to aquatic receiving environments.

Hundreds of compounds have been identified in bleached kraft pulp mill effluents (BKPMEs), and many remain unidentified due to the complexity of the effluents. Compounds identified to date include wood-derived carbohydrates, lignin derivatives, organochlorine compounds (chlorinated phenols, catechols, guaiacols, dioxin, furan, and resin and fatty acids), and extractive compounds (resin and fatty acids, phytosterols, and phenols) (Kringstad and Lindström, 1984; LaFleur and Barton, 2003; Suntio et al., 1988). BKPMEs are typically treated prior to their release to surface waters. In the 1970s and 1980s, primary treatment was common and consisted of screening and settling of solids in clarifiers or settling basins (Folke, 1996; McLeay and Associates, 1987; NCASI; 1989, Smook, 1994). In the 1980s and 1990s, after the implementation of regulations for environmental protection, many mills in Canada and Scandinavia installed secondary effluent treatment (Folke, 1996; Kovacs et al., 1996, 2003). Secondary effluent treatment is a biological process in which microorganisms in treatment plants or basins break down and detoxify biodegradable organics through cellular respiration prior to effluent release (Figure 24.3) (NCASI, 1989; Smook, 1994). This treatment process is controlled to maximize biological activity by maintaining aerobic conditions and optimal nutrient concentrations in the basins.

Secondary effluent treatment has significantly reduced the environmental impact of PMEs by reducing the amount of oxygen-consuming biodegradable organics (biochemical oxygen demand [BOD]), the concentration of chlorinated organic compounds, and acute lethality of the effluent to aquatic organisms (Kovacs et al., 1996, 2003; LaFleur, 1996; Larsson et al., 2003; McLeay and Associates, 1987; NCASI, 1989). Without question, the most significant changes in the industry have been in bleaching technology and installation of secondary effluent treatment. It is difficult to determine which of these changes was more important for improving effluent quality and reducing environmental effects, as these changes occurred simultaneously at many mills. Suffice to say that both changes were significant, resulted in immediate improvements in environmental quality, and changed the direction and focus of PME effects assessment and regulation on an international scale.

History of PME Effects on Fish

A synthesis of field, laboratory, artificial stream, and caging studies examining effects of PMEs is presented in Table 24.1, Table 24.2, Table 24.3, and Table 24.4. Environmental effects can be summarized into three historical stages, beginning in the 1950s to present day. From the 1950s to late 1970s, discharge

TABLE 24.1

Summary of Field Studies of Pulp Mill Effluent Effects on Fish

Country	Location	Species		Mill Type	Refs.
Canada	Jackfish Bay, Ontario	White sucker	*Catostomus commersoni*	Bleached kraft	Munkittrick et al. (1998)
		Longnose sucker	*Catostomus catostomus*		Munkittrick et al. (1998)
		Lake whitefish	*Coregonus clupeaformis*		
	Ontario (8+ mills)	White sucker	*Catostomus commersoni*	Various	Munkittrick et al. (1998)
	St. Maurice River, Quebec	White sucker	*Catostomus commersoni*	Bleached kraft	Bussières et al. (1998)
	Moose River Basin, Ontario	White sucker	*Catostomus commersoni*	Various	Munkittrick et al. (2000b); Janz et al. (2001)
		Trout perch	*Percopsis omiscomaycus*	Various	Gibbons et al. (1998b)
	Wapiti/Smoky River, Alberta	Longnose sucker	*Catostomus catostomus*	Bleached kraft	Swanson et al. (1994)
		Mountain whitefish	*Prosopium williamsoni*		
		Longnose sucker	*Catostomus catostomus*		McMaster et al. (2000)
		Trout-perch	*Percopsis omiscomaycus*		
	Athabasca River, Alberta	Spoonhead sculpin	*Cottus ricei*	Bleached kraft	Gibbons et al. (1998a)
	St. Francois River, Quebec	White sucker	*Catostomus commersoni*	Various	Kovacs et al. (2002)
		Smallmouth bass	*Micropterus dolomieu*		
		Tesselated darter	*Etheostoma olmstedi*		
	Fraser River	Chinook salmon	*Oncorhynchus tshawytscha*	Various	Wilson et al. (2000)
	Miramichi River, New Brunswick	Mummichog	*Fundulus heteroclitus*	Various	LeBlanc et al. (1997); Fournier et al. (1998)
		Tomcod	*Microgadus tomcod*		Williams et al. (1998)
	New Brunswick and Nova Scotia (4 mills)	Tomcod	*Microgadus tomcod*	Various	Couillard et al. (1999)
	Port Harmon, Birchy Cove, Newfoundland	Winter flounder	*Pleuronectes americanus*	Various	Khan and Hooper (2000); Khan and Payne (2002)
	Various (65 mills)	Various	—	Various	Environment Canada (1997b, 2003); Munkittrick et al. (2002); Courtenay et al. (2002); Lowell et al. (2004)
Sweden	Norrsundet	Eurasian perch	*Perca fluviatilis*	Bleached kraft	Sandström (1996); Ericson and Larsson (2000)
	Husum	Eurasian perch	*Perca fluviatilis*	Bleached kraft	Sandström (1996)
	Karlsborg	Eurasian perch	*Perca fluviatilis*	Bleached kraft	
	Munksund	Eurasian perch	*Perca fluviatilis*	Unbleached kraft	
	Lövholmen	Eurasian perch	*Perca fluviatilis*	Unbleached kraft	
	Mönsterås	Eelpout	*Zoarces viviparous*	Bleached kraft	Larsson et al. (2000); Larsson and Forlin (2002)

United States	Pigeon River, Tennessee	Redbreast sunfish	*Lepomis auritus*	Bleached kraft	Adams et al. (1992)
	Columbia River, Washington	White sturgeon	*Acipenser transmontanus*	Bleached kraft + non-pulp-mill sources	Foster et al. (2001)
	St. Johns, Florida	Largemouth bass	*Micropterus salmoides*	Bleached kraft	Sepúlveda et al. (2003); Holm et al. (2000)
	MacKenzie River, Oregon; Willamette River, Oregon; Codorus Creek, Pennsylvania; Leaf River, Mississippi	Various	—	Bleached and unbleached kraft	Hall et al. (2000)
	Not defined	Bluegill sunfish	*Lepomis macrochirus*	Bleached kraft	D'Surney et al. (2000)
	Not defined	Blue catfish	*Ictalurus furcatus*	Unbleached kraft	Felder et al. (1998)
Finland	Lake Saimaa	Perch Roach	*Perca fluviatilis* *Rutilus rutilus*	—	Karels and Oikari (2000); Karels et al. (2001)
New Zealand	Tarawera River	Rainbow trout	*Oncorhynchus mykiss*	CTMP and kraft mill	Donald (2003)
	Waikato River	Rainbow trout Goldfish Common bully	*Oncorhynchus mykiss* *Carassius auratus* *Gobiomorphus cotidianus*	—	Richardson (1997)
Portugal	Vouga River	Eel (caged)	*Anguilla anguilla*	Bleached kraft	Pacheco and Santos (1999); Ferreira et al. (2002)
Chile	BioBio River	Striped mullet Falklands mullet	*Mugil cephalus* *Eleginops maclovinus*	—	Gaete et al. (2000); Barra et al. (2001)

Source: Adapted from Munkittrick, K.R., in *Fishes and Forests: Worldwide Watershed Interactions and Management*, Northcote, T.G. and Hartman, G.F., Eds., Blackwell Scientific, Oxford, pp. 336–361, 2004.

TABLE 24.2

Summary of Laboratory Studies of Pulp Mill Effluent Effects on Fish

Species		Endpoints	Duration	Mill Type	Refs.
Exposure to Whole Mill Effluent or Process Streams					
Fathead minnow	*Pimephales promelas*	Growth, reproduction	≤180 days	Bleached kraft	Robinson (1994); Kovacs et al. (1996)
			≤200 days	Bleached kraft	Borton et al. (2000a)
			90–120 days	Bleached sulphite	Parrott et al. (2000a, 2003)
Rainbow trout	*Oncorhynchus mykiss*	MFO induction	4 days	Various	Martel and Kovacs (1997)
		Physiological, biochemical, growth	4.5 months	Bleached kraft	Mattson et al. (2001a)
		Vitellogenin, steroids, MFOs	21 days	Bleached kraft	Tremblay and Van Der Kraak (1999)
Spotted murrel	*Channa punctatus*	Immune endpoints	15–90 days	Paper mill	Fatima et al. (2001)
Stinging catfish	*Heteropneustes fossilis*	Hematology	90 days	Paper mill	Ahmad et al. (1998)
Bluegill sunfish	*Lepomis macrochirus*	Hematology	56 days	Bleached kraft	D'Surney et al. (2000)
		Organ size and biochemical	30 days	Unbleached kraft	Felder et al. (1998)
Chinook salmon	*Oncorhynchus tshawytscha*	MFO, DNA adducts	28 days	Bleached kraft	Wilson et al. (2001)
Goldfish	*Carassius auratus*	Sex steroids	≤20 days	Various	McCarthy et al. (2003); Parrott et al. (2000c)
Largemouth bass	*Micropterus salmoides*	Messenger RNA	7 days	Bleached kraft	Denslow et al. (2000)
European whitefish	*Coregonus lavaretus*	Stress responses	3–6 weeks	Bleached kraft	Lappivaara (2001)
		Vitellogenin gene	—	Various	Mellanen et al. (1999)
		Physiological parameters	30 days	Bleached kraft	Soimasuo et al. (1998b)

Roach	Rutilus rutilus	Immune system, physiological changes	21 days	Bleached kraft	Aaltonen et al. (2000a)
		Immune system	5 weeks	Various	Aaltonen et al. (2000b)
Tilapia	*Oreochromis mossambicus*	MFO	3–7 days	Bleached kraft	Chen et al. (2001)
Eel	*Anguilla anguilla*	EROD	≤3 days	Bleached kraft	Pacheco and Santos (1999)
		Stress parameters	≤188 hours	Bleached kraft	Santos and Pacheco (1996)
Common jollytail	*Galaxis maculatus*	Whole organism, MFO, histology	7 weeks to 3 months	Eucalypt-based pulp mill	Woodworth et al. (2003)
Mosquitofish	*Gambusia affinis*	External characteristics	21 days	Not stated	Ellis et al. (2000)
Mummichog	*Fundulus heteroclitus*	Organ size, sex steroids	7–21 days	Bleached kraft	Dubé and MacLatchy (2001); MacLatchy et al. (2004); Hewitt et al. (2002)
Exposure to Specific Compounds					
Rainbow trout	*Oncorhynchus mykiss*	MFOs, developmental abnormalities	4–32 days	Retene	Billiard et al. (2003); Fragoso et al. (2000); Oikari et al. (2002)
Zebrafish	*Danio rerio*	Developmental abnormalities	14 days	Retene	Billiard et al. (2003)
		Steroid levels, vitellogenin	21 days	β-Sitosterol	Tremblay and Van Der Kraak (1998, 1999)
Brown trout	*Salmo trutta lacustris*	Reproduction and offspring survival	4.5 months	Wood sterols	Lehtinen et al. (1999)
Eelpout	*Zoarces viviparus*	Reproduction and offspring survival	8+ months	Wood sterols	Mattsson et al. (2001b)

Source: Adapted from Munkittrick, K.R., in *Fishes and Forests: Worldwide Watershed Interactions and Management*, Northcote, T.G. and Hartman, G.F., Eds., Blackwell Scientific, Oxford, pp. 336–361, 2004.

TABLE 24.3

Summary of Artificial Stream or Mesocosm Studies of Pulp Mill Effluent Effects on Fish

Species		Endpoints	Duration	Mill Type	Refs.
Brown trout	*Salmo trutta*	Steroid levels; egg hatchability	4 months	Mechanical	Johnsen et al. (2000)
		Physiological, biochemical	8 weeks	Thermomechanical	Johnsen et al. (1998)
Rainbow trout	*Oncorhynchus mykiss*	Growth, gonad development, steroids, vitellogenin	2 months	Bleached kraft/TMP	van den Heuvel et al. (2002)
		Whole organism, biochemical	6–9 months	Bleached kraft	Hall et al. (2003)
Largemouth bass	*Micropterus salmoides*	Production, whole organism, steroids, MFOs	510–610 days	Bleached kraft	Borton et al. (2000b)
Bluegill	*Lepomis macrochirus*				
Golden shiners	*Notemigonus crysoleucas*				
European whitefish	*Coregonus lavaretus*	Stress responses	3 to 6 weeks	Bleached kraft	Lappivaara (2001)
		Vitellogenin gene	—	Various	Mellanen et al. (1999)
Mummichog	*Fundulus heteroclitus*	Whole organism, sex steroids	21–60 days	Various	Dubé and MacLatchy (2000a)
Longnose dace	*Rhinichthys cataractae*		60 days	Bleached kraft	Dubé et al. (2002a,b, 2004)
Stickleback	*Gasterosteus aculeatus*	Length, weight	≤126 days	Bleached magnephite pulp	Lehtinen (2003)
			16 months	Bleached kraft	Tana et al. (2003)
White sucker	*Catostomus commersoni*	Accumulation of active compounds	20 days	Bleached sulfite/groundwood	Hewitt et al. (2003b)

Source: Adapted from Munkittrick, K.R., in *Fishes and Forests: Worldwide Watershed Interactions and Management*, Northcote, T.G. and Hartman, G.F., Eds., Blackwell Scientific, Oxford, pp. 336–361, 2004.

TABLE 24.4

Summary of Caging Studies of Pulp Mill Effluent Effects on Fish

Species		Endpoints	Duration	Exposure	Refs.
White sucker	*Catostomus commersoni*	MFO	Up to 14 days	Bleached kraft	Munkittrick et al. (1999)
		Accumulation of MFO inducers, steroid ligands	4 days	Bleached kraft	Hewitt et al. (2000)
Goldfish	*Carassius auratus*	Steroids	Up to 16 days	Bleached kraft	McMaster et al. (1996b)
Crucian carp	*Carassius carassius*	Organ size, histology, steroids, vitellogenin	Living in secondary treatment pond	Bleached kraft	Kukkonen et al. (1999)
Eel	*Anguilla anguilla*	MFO and biochemical	3 days	Bleached kraft	Pacheco and Santos (1999)
Whitefish	*Coregonus lavaretus*	MFO, bile conjugates, vitellogenin, steroids	1 month	Bleached kraft	Karels et al. (1999)
		Physiological stress responses	30 days	Bleached kraft	Lappivaara et al. (2002)
		Vitellogenin gene	30 days	Various	Mellanen et al. (1999)
		Physiological parameters	30 days	Various	Soimasuo et al. (1998a)
Largemouth bass	Micropterus salmoides	Whole organism, steroids, vitellogenin	28–56 days	Various	Sepúlveda et al. (2001)
Long- and short-finned eels	*Anguilla diefenbachii* and *A. australia*	EROD, biochemical, whole organism	32–108 days	Various	Jones et al. (2003)

Source: Adapted from Munkittrick, K.R., in *Fishes and Forests: Worldwide Watershed Interactions and Management*, Northcote, T.G. and Hartman, G.F., Eds., Blackwell Scientific, Oxford, pp. 336–361, 2004.

of PMEs high in fiber and BOD resulted in habitat degradation (e.g., smothering of spawning beds due to fiber deposition, reduced oxygen concentration in the water column) and acute lethality to fish in receiving waters (Folke, 1996; McLeay and Associates, 1987; Owens, 1996). In response to these effects, regulators established "end-of-pipe" effluent quality limits for BOD and total suspended solids (TSS), an indication of the fiber concentration in effluents (Folke, 1996; Owens, 1991). Final effluents could also not be acutely lethal. The pulp and paper industry in North America and Scandinavia responded to observations of environmental impact and regulatory concern by significantly improving effluent quality with better process and spill control and installation of effluent treatment (Smook, 1994; Folke, 1996).

In the 1970s and early 1980s, the focus shifted to identification of the chemicals responsible for acute toxicity of PMEs (Folke, 1996; Owens, 1991, 1996). A landmark paper in this regard was that of Leach and Thakore (1975), who identified resin acids as primary contributors to acute effluent toxicity to fish. This led to increased attention to resin and fatty acids and chlorinated phenolics (Holmbom and Lehtinen, 1980; Kringstad and Lindström, 1984; Owens, 1991). In addition, the discovery of persistent chlorinated organic compounds (dioxin and furan congeners) bioaccumulating in aquatic biota led to regulations restricting their discharge in whole (adsorbable organic halide) or in part (dioxins and furans) (Folke, 1996; Servos et al., 1994).

During the 1980s and 1990s, process modifications largely focused on changing the type or volume of bleaching chemicals used to reduce the formation and discharge of chlorinated organic compounds (Kovacs et al., 2003; Oikari and Holmbom, 1996; Owens, 1996; Servos, 1996; Strömberg et al., 1996). Prior to the discovery of these compounds, elemental chlorine was the dominant bleaching agent. To reduce the discharge of organochlorines, elemental chlorine-free technologies were developed where elemental chlorine was replaced wholly, or in part, by chlorine dioxide. Totally chlorine-free bleaching technologies have also been developed where bleaching is conducted using oxygen, ozone, or hydrogen peroxide. The concentration of organochlorines also decreased due to reduced volumes of chemicals used in the bleach plant. Improved delignification of the pulp prior to the bleach plant reduced the amount of fiber entering the plant, which in turn, reduced the volume of bleaching chemicals required to achieve desired pulp brightness (NCASI, 1989).

Changes in mill process and effluent treatment during the 1980s and 1990s improved effluent quality and shifted the assessment of effects from measurements of lethal toxicity to the potential of effluents to cause more subtle, sublethal effects such as reduced growth and reproduction (Folke, 1996). Interest was also focused on the potential for physiological indicators to identify pulp mill effects in fish at early stages of effect diagnoses. Induction of liver detoxification enzymes (mixed-function oxygenases [MFOs]), for example, received much attention as a physiological indicator of exposure of fish to PME (Hodson, 1996; Martel et al., 1994, 1995; Munkittrick et al., 1992a,b, 1994; Oikari and Holmbom, 1996; Soimasuo et al., 1998a,b).

Long-term field studies in Sweden, Finland, and Canada recorded the changes in effects measured through periods of mill modernization. The receiving waters of a BKPM at Norrsundet, Sweden, on the coast of the Bothnian Sea, have been a site of extensive field investigations examining effluent effects on the survival, reproduction, physiology, biochemistry, histopathology, and morphology of perch (*Perca fluviatilis*) (Andersson et al., 1988; Södergren, 1989). In the 1980s, typical effects measured in the exposed fish included fin erosion, reduced gonad weight, delayed sexual maturation, impaired fry production, liver enlargement, induction of liver detoxification enzymes, affected carbohydrate metabolism, disturbed ion balance, stimulated red blood cell production, and a weakened immune system (Andersson et al., 1988; Larsson et al., 1988; Sandström et al., 1988). After significant improvements in mill process and effluent treatment, studies in 1993 indicated many of the biochemical and physiological effects in perch had either disappeared or were significantly reduced (Ericson and Larsson, 2000; Larsson et al., 2003). Delayed sexual maturity, smaller perch embryos, altered sex ratios, genotoxic effects (i.e., DNA adducts), and slight responses in liver detoxification enzymes, however, continue to be reported (Sandström, 1995; Ericson and Larsson, 2000; Larsson et al., 2000; Larsson and Forlin 2002; Larsson et al., 2003; Sandstrom and Neuman, 2003).

Since 1991, an intensive Finnish case study has been conducted at southern Lake Saimaa, in connection with major technological changes in the pulp and paper industry (Karels and Oikari, 2000). Perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) living in the lake are exposed to effluents from three pulp and

paper mills. Studies conducted after changes in mill bleaching technology showed that exposure to PME compounds, based on induction of MFO activity, exists but at much lower levels than before modernization. Alterations in sex steroid hormones, growth, gonad size, and fecundity continue to be documented although species responses differ and difficulties remain in establishing direct cause and effect linkages with the PME (Karels et al., 1998; 2001).

Field studies conducted in Canada (Jackfish Bay, Lake Superior, Ontario) from 1988 to present day have documented improvements in fish responses to PME although reproductive impairment in white sucker (*Catostomus commersoni*) and lake whitefish (*Coregonus clupeaformis*) remains (McMaster et al., 1991; Munkittrick et al., 1991, 1992a,b, 1994, 2000a). Alterations in endocrine and reproductive function include smaller gonad and egg size, increased age at sexual maturation, decreased levels of reproductive steroid hormones, and altered expression of secondary sex characteristics (McMaster et al., 1991; Van Der Kraak et al., 1992, 1998). Jackfish Bay continues to be the subject of intense study examining reproductive performance in fish (McMaster et al., 1992, 1995, 1996a; Munkittrick et al., 2003).

In addition to the Swedish, Finnish, and Canadian field case studies, additional studies have examined the potential of PMEs to affect fish. Some field and laboratory studies have shown that PMEs discharged from modernized mills affect fish reproduction (Adams et al., 1992; Dubé and MacLatchy, 2000a, 2001; Gagnon et al., 1994a,b; Karels et al., 1998; Kovacs et al., 1995; Leblanc et al., 1997; McMaster et al., 1996b; Soimasuo et al., 1998b). Other studies, however, have failed to observe reproductive effects in fish (Borton et al., 2000a, 2003; Kovacs et al., 1996; Swanson et al., 1994, 1996). Most recently, a national evaluation described the existing response patterns for fish exposed to PMEs across Canada (Environment Canada, 2003; Lowell et al., 2004). The predominant pattern was decreased gonad weight and increased liver weight, condition factor, and weight at age. These responses are believed to be indicative of some form of metabolic disruption or impairment of endocrine functioning in combination with an eutrophication effect (Environment Canada, 2003; Lowell et al., 2004). Thus, as of today, it can be concluded that reproductive effects occur in fish exposed to some PMEs, but effects are not always consistent across mills, studies, seasons, or species, and the basis for the inconsistencies is unknown.

Approaches Used to Assess PME Effects on Fish

The assessment of PME impacts on fish has a long history and has resulted in an understanding of effluent effects at different levels of biological organization. Of equivalent significance is how PME studies have advanced our understanding of experimental and statistical design and have developed novel field, mesocosm, and laboratory techniques and decision-making approaches (Dubé, 2004). These advances have benefited the industry as a whole as well as influenced effects assessment for other sectors such as metal mining and oil and gas (Ayles et al., 2004; Environment Canada, 2001). PME effects assessment is no longer at an exclusive stage of measuring effects but has progressed to a stage of determining which effects are ecologically important and require mitigation (Dubé, 2004; Munkittrick et al., 2000a).

Study Design Considerations for Fish

Any study design for assessing contaminant effects such as PME on fish should include the following (Environment Canada, 1998, 2001):

- Definition of the goals and objectives of the study
- Site characterization or description of background information for the study
- Detailed timetable for conducting the study
- Description and justification for the species, population or community selected for study
- Selection and justification for the indicators that will be measured to assess if a change has occurred
- Description of the study design and how effects will be determined (e.g., where to sample, what to measure, how frequently to sample)

- Statistical design criteria including hypotheses, statistical methods, determination of sample size (statistical significance and power analysis)
- Description of how other supporting information (e.g., changes in benthic invertebrate communities, effluent chemistry data, toxicity data) will be used in the assessment of fish effects
- Identification of the quality assurance and quality control measures that will be taken to ensure the validity of the data

One of the most difficult decisions to make before a study is conducted is to define what an effect will be and how it will be measured. It requires identification of indicators to measure at some level of biological organization and an experimental design where changes in the indicators can be assessed relative to some benchmark or baseline condition. It also requires a framework to decide what information will act as supporting data and how they will be used to assist with interpretation of effects on fish. A good starting point for information and references on indicator selection and study design trade-offs can be found in Munkittrick et al. (2000b) and Environment Canada (1998, 2001).

Pulp and paper effects assessment with fish in Finland, Sweden, New Zealand, and Canada has made a significant contribution to development of study design approaches; for example, in the Canadian EEM program, mills are required to conduct biological monitoring studies that include a fish population and a benthic invertebrate community survey. They are also required to collect supporting information through effluent and water quality monitoring studies, which include effluent characterization, water quality monitoring, and sublethal toxicity testing (Environment Canada, 1998). The fish population survey is conducted to determine if there have been changes in indicators of fish growth, reproduction, condition, and survival by collecting fish species found in exposure and reference areas and comparing measurements of length, weight, gonad size, liver size, fecundity, and egg size. In this program, *an effect on the fish population* means a statistical difference between fish population measurements taken from exposure and reference areas (control/impact study design). Fish studies have been conducted at 65 mills across Canada using core effect indicators and statistically rigorous study designs, resulting in a national synthesis of effects (Environment Canada, 2003; Lowell et al., 2004). The database from this investigation has led to recognition of key common response patterns in fish and has presented a context to begin development of critical effect sizes for each indicator.

Relevance of Other Trophic Levels

Although this chapter focuses on fish responses to PMEs as a case study, it is important to consider other trophic levels when designing a fish assessment program. Aquatic food webs include organisms at a wide variety of organizational levels, and PME assessments have been conducted focusing on lower trophic levels (e.g., algae and benthic invertebrates), as well as higher levels (e.g., small- and large-bodied fish) (Environment Canada, 1998; Munkittrick and McCarty, 1995; Munkittrick et al., 2000b). All organisms integrate the stressors within the system, and all levels of organization are capable of exhibiting measurable changes in their own right; however, the monitoring level selected must be relevant to the hypothesis to be tested and/or to the decision-making process used to manage ecosystem health. Many of the large international field programs conducted to date have been designed as multi-trophic, integrated studies that examine PME effects on algae, benthic invertebrates, and fish (Andersson et al., 1988; Larsson et al., 2000; Lowell et al., 2004; Owens, 1991; Södergren, 1989). This approach provides invaluable information on different compartments of the aquatic receiver and can build a weight-of-evidence to determine if PME effects exist, if the effects are common across trophic levels, and to assist with interpretation of the mechanism of the effects (Owens, 1991). For example, in the national synthesis of the Cycle 2 of the Canadian EEM program, a response pattern observed in biota exposed to PME included increased benthic invertebrate density and species richness, as well as increased energy storage (i.e., increased condition and liver size) and energy allocation (i.e., increased gonad size and growth) in fish (Lowell et al., 2004). Often these responses were also coincident with increased algal biomass. These results strongly suggest a nutrient enrichment effect of the effluent across trophic levels. Once a system is understood, then monitoring multiple trophic levels may be neither necessary nor cost effective

(Munkittrick et al., 2000b). For example, on the Wapiti River in Alberta, Canada, monitoring illustrated that nutrient enrichment was the dominant effect of PME discharge and increased algal biomass affected benthic invertebrate communities and fish populations from the bottom-up (Culp et al., 2004; Dubé et al., 2004). Future studies at this site now focus on establishing relationships between nutrient levels and algal biomass accrual in an effort to reduce PME effects (Cash et al., 2004).

Indicators for Assessing PME Effects in Fish

Changes in fish exposed to PMEs could be evident at all levels of organization, including biochemical, organ, individual, population, and community. Selecting the level of organization to be used for assessment purposes depends on the study objectives and management targets (Adams et al., 1992; Karels et al., 2001; Larsson et al., 2000; Munkittrick et al., 2000b). It is not reasonable to monitor all levels of biological organization and require a "zero-tolerance" for change. It is also not reasonable to monitor only at the highest levels of biological organization and determine a change is unacceptable after a species has been pushed to extinction (Munkittrick et al., 2000b). In general, as the level of biological organization increases from individual to community, the ecological relevance increases, the time lag for detecting changes relative to stressor exposure increases, and the specificity of the response decreases (Munkittrick et al., 2000b). These factors must be recognized when the level (or levels) of organization is selected for assessment purposes.

Biochemical, physiological, and pathological variables have been very useful as indicators for detecting responses in fish exposed to PMEs (see tables) (Andersson et al., 1988; Larsson et al., 2000, 2003; Munkittrick et al., 1998). These indicators are used to assess the status of important biological functions including growth, condition, and energy metabolism; liver function; reproduction; immune defense; pathology; and hematology. In the last decade, some indicators used to assess exposure to PMEs that have received attention include changes in liver weight and condition as an indication of effects on energy stores; changes in gonad weight, sex ratios, fecundity, age at sexual maturation, egg size, and sex steroid hormones to assess effects on reproduction; changes in growth as an indication of energy allocation; and changes in the induction of liver detoxification enzymes.

Current strategies recommend that a suite of indicators be measured to reflect both population and physiological functions of the individual organisms that might be affected by PME (Larsson et al., 2000; Munkittrick et al., 1998, 2000a). This information can serve to build a weight-of-evidence approach to establish what the effect of the effluent is, what types of chemicals might be responsible, and their potential mechanism of action; however, it is critical to understand that the roles of many biochemical and physiological indicators in fish are not understood, and any conclusions on the effects of effluents based on these indicators must be interpreted in that context of uncertainty.

Liver Detoxification Enzymes

In mammals, the cytochrome P450-dependent mixed-function oxidase or mixed-function oxygenase (MFO) system is responsible for initiating the biotransformation of various organic compounds, including xenobiotic contaminants (e.g., pesticides, dioxin) and endogenous compounds such as steroid hormones (Jimenez and Stegeman, 1990). Upon exposure to foreign aromatic compounds, MFO enzymes are rapidly induced, and, as a result, they can be used as biological indicators of contaminant exposure (Hodson et al., 1991; Jimenez and Stegeman, 1990). They are mentioned specifically in this chapter as they have been used extensively to assess fish responses to PME. Exposure to secondary treated BKPMEs has been shown to induce MFO enzymes (Hodson, 1996; Munkittrick et al., 1994; Oikari and Holmbom, 1996; Soimasuo et al., 1998a,b); however, enzyme induction is not always correlated with other indicators of fish health, including changes in sex steroid levels, liver size, and gonad size (Hodson, 1996; Munkittrick et al., 1994; Swanson et al., 1992). Although MFO induction has not been mechanistically linked to other indicators of fish health, it is still commonly used in conjunction with a suite of other indicators to indicate effluent exposure (Larsson et al., 2000; Martel et al., 1996). Specifics on liver detoxification enzymes can be found in other sections of this text.

Indicators of Reproductive Function

Reproductive success in fish depends on successful courtship, mating, egg development, hatching, and growth of larvae and juveniles under suitable environmental conditions (Donaldson, 1990). Regulation of reproductive endocrine function is essential to ensure reproductive success under varying environmental conditions and to maintain internal homeostasis. Interruptions or changes to the control mechanisms of the hypothalamic–pituitary–gonadal axis of fish have the potential to alter individual reproductive capability or fitness at the best (e.g., reduced gonad growth and hormone metabolism) and alter population viability at the worst (e.g., reduced fecundity, abundance) (Arcand-Hoy and Benson, 1998; Donaldson, 1990). Exposure to some chemicals (endocrine-disrupting compounds, or EDCcs) are thought to alter reproductive function by mimicking natural hormones, inhibiting the action of hormones, and/or altering the normal regulatory function of the endocrine system (Cooper and Kavlock, 1997; Crisp et al., 1998). Changes to biological indicators of reproductive success have been used in fish to assess effects of PMEs. Exposure to effluent has reduced gonad size, fecundity, and gamete size; increased age at sexual maturation; depressed levels of reproductive steroid hormones; and altered expression of secondary sex characteristics (Andersson et al., 1988; Leblanc et al., 1997; McMaster et al., 1991, 1992; Munkittrick et al., 1991, 1992a,b, 1994; Sandström et al., 1988). Although these effects are believed to be caused by compounds that interfere with the maintenance of normal reproductive endocrine homeostasis in fish (Crisp et al., 1998; Van Der Kraak et al., 1992, 1998), the mechanisms of reproductive endocrine dysfunction with PME exposure are poorly understood. Van Der Kraak et al. (1992) have shown that exposure of white sucker to BKPMEs affected reproductive endocrine communication at several sites within the hypothalamic–pituitary–gonadal axis, including gonadotropin production and ovarian steroidogenesis; however, correlations between indicators of reproductive health and with other fish health indicators are not consistent, resulting in the ecological relevance of these indicators being questioned (Kovacs, 1986; Kovacs et al., 1997). In addition, not all PMEs cause changes in indicators of fish reproduction (Swanson et al., 1994, 1996). At the present time, indicators of reproductive health in fish continue to be monitored to assess PME effects. Although the ecological relevance of these indicators may be questioned, a change does represent an alteration in normal reproductive-endocrine function (Arcand-Hoy and Benson, 1998), and this has the potential for population-level effects necessitating continued measurement of these indicators.

Monitoring Approaches

Ecological Field Assessments

Field assessments are used to establish baseline environmental conditions prior to the discharge of PME or, more commonly, to determine if an existing discharge is affecting biota. Conducting studies in the field is the most environmentally relevant approach to assessing effects under real-life conditions compared to other approaches such as laboratory toxicity tests or artificial stream studies. One approach used to assess the effects of PMEs is to examine areas exposed to effluent and determine whether there are changes in the organisms living there. This approach has been used extensively to assess pulp and paper effects on fish (Table 24.1). Typically fish are collected from a series of reference sites to determine the normal variability in health under unexposed conditions. Organisms are also collected from effluent-exposed areas at increasing distances from the PME discharge. The health of fish in these collections is then compared among sites to determine if exposed fish are different, the magnitude of the difference relative to natural variability at reference sites, and if the health improves with distance from the discharge. One of the greatest challenges in any assessment program is determining if changes due to a particular stressor (e.g., PMEs) are outside of what could be expected due to natural variability alone. Comparison of indicators between reference and exposure sites is an approach that attempts to tease out stressor-induced variability (e.g., PMEs) from natural variability (e.g., individual differences in a population of unexposed fish).

Several assumptions are associated with conducting ecological field assessments to measure pulp and paper effects, including: (1) the sites sampled differ only in exposure to effluent (i.e., habitat differences do not exist); (2) adequate reference sites exist for comparison; (3) organisms are present and abundant

at all sites for collection of adequate numbers of samples; (4) there are no other effluent discharges or anthropogenic influences (i.e., municipal sewage) that are confounding interpretation of mill-related effects; and (5) the organisms are exposed (i.e., fish do not move between reference and exposed areas). Most of these assumptions are easier to meet for benthic invertebrates. For fish, field designs often involve a compromise between optimizing the study design in an effort to meet these assumptions while striving to obtain the most relevant and interpretable data.

An example of how ecological field assessments can be optimized to meet assumptions with respect to effluent exposure is selecting sampling sites upstream and downstream of pulp and paper mills that are also upstream and downstream of dams (Munkittrick et al., 2000b). This design ensures that fish do not move between reference and exposed areas. In this example, however, damming of waters presents another set of potentially confounding factors, such as the creation of reservoir areas that act as nutrient or contaminant sinks that can interfere with understanding cause and effect linkages between changes in fish and PMEs. Sample sites can also be located upstream and downstream of other confounding effluent discharges in an attempt to isolate PME-specific effects (Dubé, 2003).

Exposure of fish to PMEs can be assessed by conducting plume delineation studies and measuring tracers of effluent exposure in fish tissues (Environment Canada, 1998). Tracers in fish bile (e.g., resin and fatty acids) or fish tissues (e.g., chlorinated organic compounds, MFO induction, stable isotopes of carbon, nitrogen, and sulfur) are commonly used (Dubé, 2004). In systems that lack natural or manmade barriers to movement, the tendency in Canada has been to move toward the collection of small-bodied species of fish that have a more localized home range compared to larger, migratory species (Gibbons et al., 1998a,b). These studies have demonstrated the effectiveness of forage fish for measuring responses to PME and have also improved our fundamental understanding of species biology and life history.

Despite the use of these strategies to optimize study designs for field assessments with fish, it may not always be possible to clearly differentiate the relative contributions of specific effluents to field responses. In many field studies, the concentration and duration of exposure to effluent are difficult to quantify because of complex effluent dispersion in the water column, mobility of fish in and out of the effluent plume, and the unavoidable presence of other effluent discharges (Langlois et al., 2003; Larsson et al., 2003). In other cases, field sampling might be too dangerous, logistically demanding, or excessively expensive to implement. In these situations, caging studies and artificial stream approaches have been developed to isolate the potential impacts of effluents while simulating receiving environment conditions.

Laboratory Toxicity Tests

Laboratory tests of acute and chronic toxicity are a common approach used to evaluate the effects of PMEs on aquatic organisms (Adams, 1990). In many countries, tests are required under legislation to characterize effluent quality (Folke, 1996). Toxicity tests are used to determine if there is the potential for effects to exist, to provide quick answers to potential benefits during mill process changes, or to track toxicity during toxicity identification evaluation (TIE) procedures. Sublethal toxicity testing can also play a key role in evaluating whether potential receiving environment concerns are not being expressed under current environmental conditions.

Acute and Chronic Toxicity Tests

Acute or lethal toxicity tests involve short-term exposure of an organism to a serial dilution of effluent to determine the concentration at which the tolerance of the organism is exceeded (mortality) (Rodier and Zeeman, 1994). The most commonly employed acute toxicity test used to assess PME is the 96-hour rainbow trout (*Oncorhynchus mykiss*) test. This test is a static acute test conducted on serial dilutions of final effluent (e.g., 6.25%, 12.5%, 25%, 50%, 100%). After 96 hours of exposure, the median lethal concentration that produces 50% mortality in the test organism (LC_{50}) is determined based on comparisons with a control. Specific details of test conditions, reference toxicants, and endpoint calculations can be found in Environment Canada (1990). Chronic or sublethal toxicity tests also involve exposure of an organism to a series of effluent concentrations, but the exposure duration is longer and response endpoints are subtler (e.g., changes in growth and reproduction) (Rodier and Zeeman, 1994). Chronic toxicity testing is practiced for prevention; that is, before a species is adversely affected by effluent

exposure, some biological response to the toxicant should occur and will be observable earlier, or at a lower effluent concentration, than the acute endpoint. Sublethal toxicity tests can be short term or long term. Short-term tests are partial life-cycle tests that measure the effects of effluents on critical life stages. Long-term tests refer to full life-cycle exposures.

Two commonly employed chronic tests used to assess PME effects on fish are the fathead minnow (*Pimephales promelas*) freshwater test and the inland silverside (*Menidia beryllina*) marine test. The fathead minnow test measures the effects of effluents on larval growth and mortality after 7 days of effluent exposure (Environment Canada, 1992b). The inland silverside survival and growth test is a 7-day test where young fish (7 to 11 days old) are exposed to serial dilutions (five concentrations) of final effluent from 6 to 100% to determine survival and growth (determined by weights) effects compared to a control (USEPA, 1988). Growth effects are estimated using linear interpolation to determine the IC_{25}, the concentration (% v/v) at which growth is inhibited by 25% compared to control fish. More recently, MacLatchy et al. (2004) have developed a short-term (7-day) exposure bioassay to evaluate reproductive endocrine responses in a northern Atlantic saltwater fish, the mummichog (*Fundulus heteroclitus*). This bioassay has been used to determine the efficacy of process changes for improving effluent quality and to drive the fractionation and compound identification of individual waste streams from a pulp mill in New Brunswick, Canada. The merits of acute and chronic toxicity tests are in their ability to rapidly assess effluent quality from a biological perspective using a relatively cost-effective, standardized approach with well-defined response endpoints (Rodier and Zeeman, 1994). Toxicity testing of PMEs on fish has been used to assess the efficacy of effluent treatment in removing toxicity, to identify and control in-plant sources of toxicity, and for routine monitoring of effluent quality (Kovacs and Megraw, 1996; Kovacs et al., 1996, 2003; McLeay and Associates, 1987). Threshold concentrations of PME that are capable of causing effects have also been estimated from toxicity tests and compared to effluent concentrations found in receiving waters. Using toxicity tests to assess field effects can be useful providing the imitations of this approach are recognized (Kovacs et al., 1996). The main limitation is a lack of environmental relevance because toxicity tests are conducted in the laboratory under tightly controlled conditions with a single species. Other limitations include lack of consideration of additive or synergistic effects of other chemical stressors or in combination with natural stressors (e.g., diurnal temperature changes). The relevance of the results to other species and higher levels of biological organization (e.g., populations) in complex field conditions has also not been adequately investigated (Kovacs and Megraw, 1996; Kovacs et al., 1996). Robinson et al. (1994) reported that partial life-cycle tests using fathead minnow could not predict field effects.

Life-Cycle Exposures

The predominant long-term sublethal toxicity test used with PMEs is the fathead minnow life-cycle test (Kovacs et al., 1996; NCASI, 1985, 1998; Parrott et al., 2003; Robinson et al., 1994). In this test, fathead minnow are exposed from the egg stage to sexual maturity and reproduction. Endpoints measured include egg hatching, survival and growth of the parent generation, and hatching and larval survival of the first generation of offspring (F_1). Experimental duration for a single life cycle is typically around 200 days. Primary advantages of a life-cycle test are that the effects of effluent exposure can be followed through key developmental stages of a species under controlled laboratory conditions. In addition, these tests allow for an evaluation of the population-level and multigenerational consequences of effluent exposure. Although a variety of indicators are measured in these tests, application for pulp and paper effects assessment has focused on fish reproductive health. Results from fathead minnow life-cycle tests show that some indicators of fish reproduction are affected by PME exposure but at concentrations higher than those found in receiving environments (Borton et al., 2003; NCASI, 1985; Parrott et al., 2000a; Robinson, 1994). In addition, these effects do not consistently transfer to subsequent generations (Borton et al., 2000a). Life-cycle tests have also been used to illustrate that mill process changes can reduce reproductive effects on fish (Borton et al., 2003; Kovacs et al., 1995, 1996). Several studies have also used fathead minnow life-cycle tests to determine the relevance of various biological indicators as predictors of fish reproductive health after PME exposure. The intention is to develop a short-term assay for assessing effluent effects that is predictive of longer term population consequences. NCASI (1985),

Robinson (1994), Kovacs et al. (1995), and Kovacs and McGraw (1996) reported that egg production (fecundity) in fathead minnow was the most sensitive indicator of fish reproductive health; however, subsequent studies by Borton et al. (2003) showed that, out of 12 bioindicators measured, only time to spawning (delayed by an average of 10 to 14 days after exposure to PME) was correlated with reduced egg production. Parrott et al. (2003) found changes in growth and secondary sex characteristics in fathead minnows exposed long term to final effluent from a bleached sulfite/groundwood mill. Males began to exhibit ovipositor formation in concentrations as low as 3.2% (v/v), and at higher concentrations most males externally resembled females. With females, masculinization as tubercle and dorsal fin dot formation was also noted at 3.2% (v/v) and above. Changes in secondary sex characteristics were the most sensitive indicator of exposure in these tests, which were 4 months in duration (Parrott et al., 2003).

Artificial Stream (Mesocosm) Studies

Field-based artificial stream systems have been used in various forms over the past 30 years to study algae, benthic invertebrates, and fish (Lamberti and Steinmen, 1993). Studies using fish have examined growth responses, reproductive behavior, competition, predation, energetics, habitat use, and ecotoxicology (Gelwick and Matthews, 1993). Artificial streams have also been used to assess the effects of PMEs on fish (Borton et al., 1996; Dubé et al., 2002a,b; NCASI, 1983, 1989, 1993). Artificial streams have been used for PME assessments in Scandinavia (Tana et al., 2003), the United States (Borton et al., 1996; NCASI, 1993), and Canada (Culp et al., 1996, 2004; Dubé et al., 2002a,b, 2004) (Table 24.3). The strength of using field-deployed systems lies in their ability to control exposure conditions while maintaining some environmental realism. Field-based artificial streams control the type, duration, and concentration of effluent exposure while maintaining ambient conditions of water temperature, water quality, and photoperiod (Culp and Podemski, 1996; Culp et al., 1996; Dubé et al., 2002a).

In southwestern Finland, significant work has been conducted examining structural and functional effects of natural factors (e.g., light, nutrients, temperature) and PME on aquatic ecosystems (Lehtinen, 2003). This work began in 1991 using multispecies brackish water littoral mesocosms consisting of open-air pools (8 m^3) supplied with a continuous flow of brackish water from the Baltic Sea (Lehtinen, 2003; Tana et al., 1994).

The National Council for Air and Stream Improvement (NCASI) in the United States has also used outdoor experimental streams to assess the effects of PMEs (Borton et al., 1996; NCASI, 1983, 1989, 1993). These streams have been in use since 1968 to assess various aspects of PME exposure on different aquatic species. The streams consist of 100-m in-ground channels that have been modified to represent different fish habitat types (pools and riffles) and water depths. Some streams are allocated as controls and others are exposed to effluents. Studies are typically long-term involving exposure durations of close to a year.

In Canada, various systems have been developed since 1991 to measure PME effects on benthic food webs (Culp et al., 1996, 2000; Dubé and Culp, 1996). In the late 1990s, the technology developed to assess the effects of PME on small-bodied fish (Dubé et al., 2002a,b). To provide an example, one system consists of 16 circular tanks or streams on two trailers (Figure 24.4) (Culp and Podemski, 1996; Culp et al., 1996; Dubé and MacLatchy, 2000a). The tanks are 1 m in diameter and hold a volume of 227 L. The trailers are situated near a surface water source that is not exposed to PME (i.e., reference site). Natural receiving water is pumped into a head tank and then through a distribution manifold and is then delivered to each tank at a controlled rate. PME is also delivered to each tank at a specified dilution. Each stream is then seeded with substrate and benthic invertebrates and/or fish endemic to the receiving environment being studied. Experiments are conducted over a desired exposure period (typically 30 to 60 days) and biological endpoints are measured. In Canada, the most recent phase of development has occurred since 1999 where multiple systems have been used simultaneously to assess the effects of PME on benthos, fish, and self-sustaining aquatic food webs (Dubé, 2004) (Figure 24.4). These advances in artificial stream technology have provided information on effects and stressor sources that facilitated interpretation of biotic responses measured in the field (Culp et al., 2000). These findings also contributed to the acceptance of this technology as monitoring alternatives for the Canadian EEM Programs (Dubé et al., 2002a). Disadvantages of artificial stream techniques include divergence of

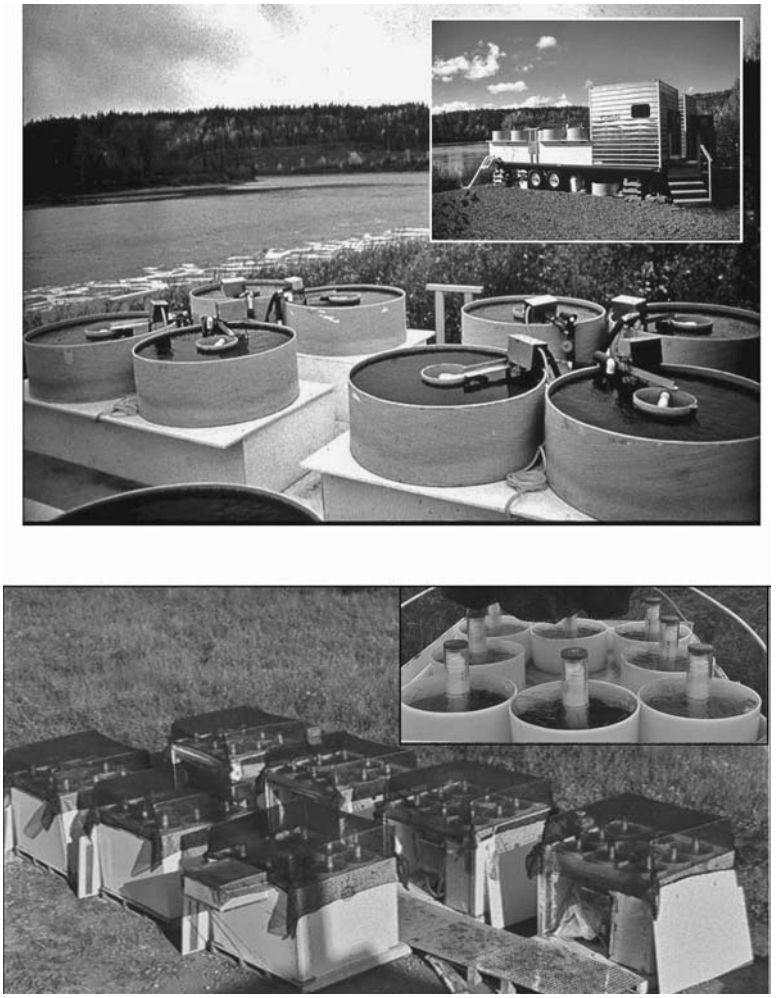

FIGURE 24.4 Examples of mesocosm systems used to assess the effects of pulp and paper effluents on fish.

replicate streams after long-term exposures, species use limitations for some designs, and logistical difficulties (e.g., no access to power or reference water).

Caging Studies

Until recently, caging studies have not been recommended for measuring PME effects on fish because the effects of the effluent could not be isolated from effects due to confinement stress (Environment Canada, 1997b); however, in a number of situations caging of fish has been used successfully to document exposure and to document the characteristics of biochemical responses to exposure (Birtwell et al., 1998; McMaster et al., 1996b; Munkittrick et al., 1999; Parrott et al., 2000b) (Table 24.4). For example, Birtwell et al. (1998) held juvenile Pacific salmon in 50-L net cages placed adjacent to the effluent discharges from coastal pulp mills in British Columbia, Canada, to assess effluent effects on survival. In these experiments, survival of fish was related to exposure to the effluent of the mill and related variables. Confinement of juvenile salmon did not affect short-term (4- to 12-day) survival at reference sites; therefore, caging studies should be considered as an alternative for short-duration exposures of small-bodied species or juveniles and in field situations where effluent exposure is well defined. Caging studies have also had some applications in conducting short-term exposures used to investigate the causes of field effects (see Bioaccumulation Model section below).

Attempts to Identify the Causes of Impacts Associated with PMEs

Significant investments have been made by the pulp and paper industry in process control, bleaching technology, and secondary effluent treatment which have greatly reduced but not removed environmental effects (Munkittrick et al., 1998, 2002). It is important to note that the process changes implemented by the pulp and paper industry during the 1990s have largely accomplished what they were designed to do: to reduce acute toxicity, BOD, total suspended solids loadings, and discharges of organochlorine compounds in effluent. The persistence in impacts on endocrine function is an observation that was first noted in research conducted at a small number of mills (Munkittrick et al., 1992b, 1994) and has since been validated on a national scale through analysis of EEM data (Lowell et al., 2004). In conducting research projects at selected mills and in implementing the study designs on a national scale through the EEM program, advances have been made in developing approaches for identification of stressors that are responsible for the persistent effects (nutrient enrichment, reproductive changes) in fish (Dubé, 2004). Investigation-of-cause approaches generate information on effects at increasing degrees of resolution with the goal of providing the information necessary for mitigation.

Hewitt et al. (2003a, 2005a) recently described several levels of effort that can be undertaken for cause identification related to PME effects. Although the knowledge of specific causes of environmental effects increases as one progresses through the levels of investigation, there is a concomitant increase in effort and cost. As Dubé and MacLatchy (2001) demonstrated, it may not be necessary to progress through the entire framework and identify causative compounds in order to mitigate effects. The Hewitt et al. (2003a) framework includes levels to define whether there is an effect, whether it is related to the effluent discharge facility, and whether response patterns in the receiving environment are characteristic of a particular stressor type. The next tier of the framework involves investigating individual process wastes within the mill to determine the components contributing to final effluent effects. Due to the complexity encountered in trying to isolate and identify the chemicals responsible for the impacts, isolation of process streams provides a target for more specific chemical evaluations and evaluation of stream-specific treatment options.

In a modified version of their earlier framework, Hewitt et al. (2005a) defined pathways of investigation of cause related to pulp and paper effects that are based on response patterns observed in biota below pulp mills. The consistency, extent, and magnitude of response patterns in fish and benthos are first described prior to investigating cause. The patterns themselves are then used as a first basis with which to select different types of investigation of cause. Indeed, the information gleaned from the response patterns themselves indicates which kinds of contaminants are present and their potential sources (e.g., eutrophication from inefficient nutrient management of secondary treatment). Causal investigations are subdivided into those focused on eutrophication and those associated with a contaminant effect (including metabolic disruption) (Hewitt et al., 2005a). In the case of nutrient enrichment, causal investigations adopt approaches to isolate the causal nutrients and what threshold levels are involved with specific enrichment parameters in biota. In the contaminant-focused causal investigations, questions progress along a continuum that progresses from first asking if the source within the mill can be identified to the compound classes involved and finally to identification of the specific chemicals associated with the effects. In both pathways, the fundamental question driving the investigations is whether sufficient information has been generated to define the effect such that a mitigative solution can be found.

Isolation of Causative Process Streams

The purpose of this type of source identification is to specify waste streams within the manufacturing or treatment process that are responsible for the observed effects (Dubé and MacLatchy, 2000a,b, 2001; Dubé et al., 2002b; Hodson et al., 1997; Martel et al., 1997; Parrott et al., 2000c). A variety of approaches have been tried, ranging from simple, on-site static exposures involving containers of waste from different sources within the mill (Parrott et al., 2000c) to flow-through, on-site mesocosm exposures to investigate waste streams selected by acute toxicity tests (Dubé and MacLatchy, 2000a,b, 2001). These approaches originated in an effort to circumvent the complexity of final effluents and to identify common sources of bioactive substances between mills employing various production types. Munkittrick et al. (1994),

for example, coordinated a survey of fish responses at multiple mills and concluded that responses of MFO induction, reduced gonad sizes, and altered steroid hormone levels could be seen at mills with a variety of process types and at some sites with secondary effluent treatment. Additional studies examined the relationship between MFO induction and effluents from kraft mills with different bleaching and effluent treatment technologies (Martel and Kovacs, 1997; Martel et al., 1996, 1997; Williams et al., 1996). Results showed that MFO-inducing potential could not be linked to a particular bleaching or pulping process. Induction was present in unbleached kraft mills, bleached kraft mills with different bleaching sequences, thermomechanical mills, and chemithermomechanical mills. In addition, induction was not correlated to typical compound classes including resin and fatty acids, dissolved organic carbon, adsorbable organic halogen (AOX), or other chlorinated organics. These results suggested that the source of the responsible compounds was independent of the bleach plant on the pulping side of mill operations (Folke, 1996; Kovacs and Megraw, 1996; Munkittrick et al., 1998). Williams et al. (1996) speculated that the origin of the inducing compounds was residual lignin liberated from the cellulose in either the bleaching process (through oxidation) or in the pulping process and that the discharge of pulping liquors (i.e., weak black liquor) could be a source of inducers in mills.

Examination of specific mill process streams by Martel et al. (1997) identified contaminated condensates produced when weak black liquor is reduced through evaporation in the chemical recovery process, as a major process source of MFO-inducing substances. Hodson et al. (1997) and Schnell et al. (2000) identified weak black liquor from softwood pulping as a primary source of inducing compounds. Artificial stream studies conducted by Dubé and MacLatchy (2000a) before and after a process change (reverse-osmosis treatment of condensates) showed that sex steroid depressions were removed in an estuarine killifish (*Fundulus heteroclitus*) after the process change. This result suggested that condensates were also a source of steroid-disrupting compounds. Acute and sublethal toxicity testing (Dubé and MacLatchy, 2000b) and laboratory exposures (Dubé and MacLatchy, 2001) using these mill waste streams confirmed that condensates were a source of steroid-disrupting compounds, and reverse-osmosis treatment removed the effect (Dubé and MacLatchy, 2001).

A similar waste stream assessment approach was used by Parrott et al. (2000c) in an attempt to isolate the origin of reproductive effects from effluents discharged from a bleached sulfite mill and a bleached kraft mill. Exposure of goldfish for 21 days showed that final effluents at 100% caused sex steroid disruptions, but the source of the effects could not be identified. Subsequent goldfish exposures after a process change at the bleached sulfite mill showed that the steroid-disrupting effect was removed (Parrott et al., 1999). The nature of the process change was not reported. Studies on the Miramichi River in New Brunswick, Canada, also showed endocrine disruption activity in mummichog (*Fundulus heteroclitus*) exposed to an environmentally relevant concentration of secondary treated final effluent. Sex steroids concentrations were reduced in these fish to a level greater than that observed with exposure to primary treated effluent at the same concentration, suggesting that secondary treatment itself was a possible source (Dubé et al., 2002b). Although this waste stream approach has been effective at isolating sources of compounds causing MFO induction and sex steroid depressions in fish, results are very mill specific. Each pulp mill is unique in its handling of waste streams; thus, a detailed understanding of mill operations and mill-to-mill differences in operations is required before the relevance of these results among mills can be determined. Further studies are underway in an effort to identify the source of the reproductive effects at these mills.

Characterization and Identification of Causative Compounds

The last tiers of the Hewitt et al. (2003a; 2005a) framework relate to characterizing the chemical classes involved in the effect and, ultimately, identifying the specific chemicals associated with the responses. The exposure profile of the effect can indicate several properties of the chemicals; for example, if the response in aquatic biota occurs rapidly upon exposure, it indicates the responsible compounds are readily bioavailable. To illustrate this concept, we present a caging study by Munkittrick et al. (1999) implemented to understand the characteristics of the PME compounds responsible for MFO induction. MFO activity was induced after 3 to 4 days. In addition, (1) uptake of the inducer was very rapid in

field trials, and MFO induction peaked within 2 days in warm water; (2) the inducers at some mills could be excreted rapidly by fish, but excretion involved an inducible mechanism which required greater than 4 days of exposure; and (3) at Jackfish Bay in Ontario, it seemed likely that the inducers were not accumulated from the food chain, although this historically may have been a component.

Various methods and approaches have been used to isolate bioactive substances from PMEs. Despite the efforts of several investigators, limited information is available on the identities of compounds present in final effluents that are associated with reproductive abnormalities in wild fish (Munkittrick et al., 2002). This is due to a number of factors, such as the chemical complexity of effluent discharges, a lack of understanding of the mechanisms involved, and differences in the response patterns of wild fish and laboratory species used to conduct investigations (Van Der Kraak et al., 1998).

The last tiers of the framework reduce this seemingly overwhelming task into achievable components by asking a progressive, more detailed set of questions regarding the chemical identities of the bioactive substances. In the case of EEM, this is done primarily to provide investigators with discrete steps of information such that causal investigations may be halted when sufficient information is gained (Hewitt et al., 2005a). A primary means by which to achieve this goal is through implementation of toxicity identification evaluations (TIEs), which provide guidance in the identification of substances causing toxicity in complex mixtures (USEPA, 1991, 1993a,b, 1997).

Toxicity Identification Evaluation Procedures

The TIE approach uses the responses of organisms or appropriate bioassay surrogate to detect the presence of active chemicals in a complex mixture. TIEs characterize the active substances of interest in three phases, and they were developed for municipal sewage in concert with toxicity reduction evaluations (TREs) to ameliorate effluent acute and chronic toxicity. Modifications of this approach have been used to investigate hormonally active substances associated with metabolic disruption in fish exposed to PMEs, and it is possible to investigate any activity of interest by selecting a different endpoint. The first phase of a TIE involves: (1) determining the characteristics of the active agents and (2) establishing whether or not the effect is caused by the same substances. Failure to establish effect variability related to the active substances could lead to erroneous conclusions and control measures that do not eliminate the effect. The physicochemical properties of the active substances can be described using effluent manipulations coupled to a bioassay that either duplicates the field effects or is mechanistically linked to them. Each test is designed to alter the substances themselves or change their bioavailability so that information on the nature of the substances can be obtained. Repeating these tests over time on the same sample will provide information on the consistency of the substances to cause the effect. Examples of effluent manipulations include filtration, pH adjustments, addition of oxidizing agents and chelating agents, temperature adjustments, aeration, and solid-phase extraction. If relatively simple modifications of this stage remove the effect during testing, it may be possible that the investigation can be halted at this juncture and these manipulations employed on an industrial scale.

What Are the Chemical Classes Involved in the Effect?

The first phase of a TIE involves specific methods to isolate the active chemicals and propose structures for their identification. In this step, further separation of active components from inactive substances is likely necessary for their identification and confirmation. These methods are specific to the classes of chemicals outlined above and utilize bioassay responses to evaluate the success or failure of extraction, separation, and concentration steps in isolating the bioactive substances. The question of whether one or more bioactive substances are involved complicates this process, and the solution to this problem is to focus on the active component that is easiest to identify. Examples of isolation techniques include solid-phase extraction, high-performance liquid chromatography (HPLC), and solvent extraction. Chemical isolation steps proceed in an iterative fashion, directed by bioassay responses until either further isolations are not possible or candidate chemicals are identified. Once there is strong evidence that one or more candidate chemicals are associated with the response, the last phase can be initiated.

What Are the Candidate Causative Chemicals Associated with the Effect?

This step involves techniques that confirm the proposed substances are in fact responsible for the observed toxicity. This is usually accomplished through a weight-of-evidence assemblage of information that collectively establishes the identity of the active compounds. It is also equally important to establish that the cause of the effect is consistent over time so that amelioration efforts can adequately address the effect. Some judgment must be exercised in terms of the extent to which confirmatory tests are carried out, which reflects the authenticity of the results. As an example, if a suspected substance can be removed by inexpensive pretreatment or process modification, a higher level of uncertainty may be acceptable than if an expensive treatment plant is required.

Can the Candidate Chemicals Be Confirmed to Cause the Effect?

Complete confirmation of isolated chemicals proposed as causative agents is challenging in that it requires procurement of authentic standards for chemical and toxicological verification. It is possible that authentic standards of candidate structures will not be commercially available and custom synthesis may be required. Custom synthesis can be expensive, time consuming, and, depending on the structure, difficult to carry out. In the absence of complete confirmation it is important to recognize that valuable information regarding the chemical characteristics of the active compounds will nevertheless be derived from all previous work. With this information and tentative chemical structures it may be sufficient to tentatively assign cause and proceed on confirmatory approaches that may include:

- *Correlation approach*—A strong consistent relationship between the concentrations of the suspected agents and the bioassay response can be established.
- *Symptom approach*—Different active substances often produce different symptoms in response. By comparing exposures of the effluent sample to those of pure suspected active substances, one can obtain further evidence regarding whether or not the suspected agents are responsible. Examples of symptoms include species sensitivities, shapes of dose–response curves, and time for the effect to occur.
- *Spiking approach*—Suspected agents are added to the effluent to determine if a proportional response in the bioassay is obtained.

Bioaccumulation Model

Applying TIEs to PMEs has proven to be problematic for the reasons outlined above. As a result, an alternative strategy to investigating complex mixtures using PMEs as a model has been developed (Parrott et al., 2000b; Hewitt et al., 2000, 2003b, 2004, 2005b). This approach utilizes controlled fish exposures to final effluents and investigates tissue burdens of bioactive substances to determine what compounds are bioavailable to fish and therefore of the most relevance. In the development of this model, it has been shown that under high exposure conditions (50% v/v), multiple ligands for fish sex steroid receptors and the aryl hydrocarbon (Ah) receptor are readily bioavailable to fish exposed to effluent from a bleached kraft mill (Hewitt et al., 2000) and a bleached sulfite/groundwood mill (Hewitt et al., 2003b). Further studies have validated the accumulation model to show that under spring conditions of high dilution at a third bleached kraft mill, fish accumulate hormonally active substances and that there are gender-specific differences in patterns of accumulation (Hewitt et al., 2004, 2005b). These studies were conducted at sites where wild fish or fathead minnow tests have demonstrated clear effects on the reproductive endocrine system, thereby providing a mechanistic linkage to the exposure of these compounds at these sites. The investigation of active substances in tissues considers one of the basic axioms of toxicology: The response of an organism is the result of a sufficient dose at the site of toxic action. Conducting characterizations of unknowns in highly complex mixtures in this manner considers multiple exposure pathways that would be ignored in direct investigations of final combined effluent or process stream investigations: (1) the modification or creation of active compounds in secondary treatment, (2) the modification or creation of active substances in the receiving environment, (3) the metabolic activation of inactive precursors within the organism, and (4) the ability of organisms to excrete the compounds by an inducible mechanism to reduce the effective dose at the target site.

Status of Identification of Responsible Chemicals

Despite the various approaches used to identify the bioactive compounds in PMEs, the compounds responsible for the continuing reproductive changes in fish at some mills have remained elusive. The initial uncertainty regarding the role of chlorine bleaching and dioxins in fish responses was resolved by the mid-1990s, when it was determined that effects were not correlated with effluent adsorbable organic halogen (AOX) levels and that releases of dioxins had decreased substantially (Munkittrick et al., 1998; Owens, 1996; Sandström, 1995). In the mid-1990s, researchers were able to partially attribute induction of detoxification enzymes in fish to wood components (Hewitt et al., 1996; Schnell et al., 2000; Williams et al., 1996). Other studies, however, did not show direct correlation between indicators of exposure (e.g., EROD activity) and primary wood components, such as resin acids (Ferguson et al., 1993). Similarly, the observation that reproductive effects occurred in fish near mills with different bleaching technologies suggested that the responsible compounds might be natural wood compounds as opposed to cooking or bleaching chemicals (Hall and LaFleur, 2003; LaFleur and Barton, 2003; Van Der Kraak et al., 1998). Some tannins, natural wood compounds that form the defense mechanism of a tree to ward off insect infestations, and plant phytosterols are present in PMEs (Cook et al., 1997; Hall and LaFleur, 2003; Van Der Kraak et al., 1998). Structurally, phytosterols have the potential to function as hormone mimics affecting reproductive steroid production and function (Biermann, 1996). Some studies showed that individual wood extractives (e.g., β-sitosterol) have the potential to affect fish reproduction (Lehtinen et al., 1999; MacLatchy and Van Der Kraak, 1995; Van Der Kraak et al., 1998) (Table 24.2); however, a lack of correlation between threshold reproductive responses and effluent concentrations of individual wood species in effluents (e.g., plant sterols) suggested that additional unidentified compounds and mechanisms were involved (Munkittrick et al., 1998). By the late 1990s, source identification approaches, in concert with the development of mechanistically linked *in vitro* and *in vivo* bioassays, have shown that multiple compounds in PMEs are affecting fish (Dubé and MacLatchy 2001; Hewitt et al., 2002; McMaster et al., 1996a; Van Der Kraak et al., 1992). Furthermore, it is highly likely that the complexity of the responses is related to single chemicals exerting their effects in multiple pathways, some of which are bound to interact with each other in the form of synergism and antagonism.

Thus far, accumulation studies have shown that bioactive substances are bioavailable and accumulated rapidly, consistent with the body of evidence that has shown that a sustained exposure is required to cause both elevated enzyme activity and depressions in sex steroid levels. The patterns of these substances in effluents and fish tissues are not correlated with mill production type or effluent treatment. Collectively, these findings show that bioactive substances originate from wood and are derived from lignin or terpenoids. They are liberated during pulp digestion, and in kraft mills they are present in black liquor and chemical recovery condensates (Dubé and MacLatchy, 2000a,b, 2001; Hewitt et al., 2005c; Martel et al., 1997; Schnell et al., 2000). Additional bioactive substances are also present in bleachery effluents containing residual lignin. Certainly, research in this area over the next decade will bring us closer than ever to understanding why fish continue to be affected by PMEs and identifying the compounds responsible for the effects.

Decision Making Relative to Pulp Mill Impacts

One of the greatest challenges in the next decade will be incorporating the science of PME effects assessment into decision-making frameworks for stakeholders to assess how important an effect is, once it is measured, and in the context of socioeconomic factors. With respect to the pulp and paper sector, it has been difficult to obtain consensus among multistakeholder groups on definitions of what constitutes an effect, when a response is an impact, and when an impact becomes damage. As an example, after 12 years of studies conducted on the potential impacts of PMEs at Jackfish Bay, Lake Superior, Ontario (Munkittrick et al., 1998), there is no consensus on whether impacts exist. The controversy rests on whether the delayed maturity and altered gonadal sizes observed at this site represent impacts. This lack of consensus is not unique to PME effects assessment and has been observed for many different anthropogenic stressors; however, the pulp and paper sector is in a unique situation. Due to the extensive

collection of data, PME effects assessment is no longer focused on how to measure effects but has progressed to a stage of determining which effects are ecologically important (Munkittrick et al., 2000a).

One of the major factors limiting progress with regard to the interpretation of effects is the misconception that all impacts are adverse, and therefore are unacceptable. As Munkittrick et al. (2000b) pointed out, individuals can survive numerous biochemical impacts, and populations can survive numerous individual impacts. Effects need to be evaluated with appropriate perspective and in the context of sustainable development. Consideration of the magnitude of a change and the consistency of a change among indicators (e.g., liver size and condition in fish) and across trophic levels (e.g., benthos and fish effect indicators) will be critical to assist with assessing the importance of a change. The use of power analyses to develop confidence in a measured change will also be an important component to the assessment process. Determining the extent to which the change is relative to natural variability measured at reference sites is also important.

National-scale monitoring programs have provided invaluable information on the types of response patterns observed in receiving environments exposed to PMEs and the magnitude of the changes across mills (Environment Canada, 2003; Lowell et al., 2004). This information provides an industrywide picture to evaluate what the dominant responses are and where responses are of the greatest magnitude. These data are also serving to advance our understanding of critical biological effect sizes.

Finally, it will be necessary to evaluate changes in receiving environment quality due to PME exposure relative to other natural and manmade influences to assess the importance of PME effects. Effective management of aquatic systems requires a holistic understanding of changes resulting from the accumulation of stressor types, including land use, other industrial discharges, urbanization, and climate change. Cumulative effects assessment approaches at watershed and regional scales such as those proposed by Culp et al. (2000), Munkittrick et al. (2000b), and Dubé (2003) may provide advancement in this area. In Sweden, decisions on environmental quality objectives may be based on ethical concerns and public perceptions and not strictly on ecological relevance (Environmental Sweden, 1997). It is the responsibility of the scientific process to objectively quantify and provide information on the types of changes, the distribution of changes, the frequency and duration of changes, and the relevance of changes to other levels of organization so that this information can be used to make more informed decisions.

Conclusions

Over the past 30 years, examination of the effects of PMEs on fish has advanced our understanding of fish toxicology and has increased our awareness of the issues of managing aquatic ecosystems receiving industrial effluent discharges. Historically, pulp and paper effects on fish were documented, regulations were implemented, and the industry responded with process changes and effluent management to significantly improve effluent quality and reduce the effects of effluents on wild fish. As a result of the culmination of studies, new monitoring techniques and biochemical endpoints have been developed over the years to better assess effluent effects. Currently, research focuses on identification of mill sources and causative compounds that may be contributing to residual effects of mill effluent on fish reproduction at some mill sites. In addition, research is being conducted to develop decision-making frameworks for improved integration of effects-based science with management issues pertaining to pulp and paper effects assessment for aquatic ecosystems.

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25

Estrogenic Effects of Treated Sewage Effluent on Fish: Steroids and Surfactants in English Rivers

Charles R. Tyler, Edwin J. Routledge, and Ronny van Aerle

CONTENTS

Introduction: How Estrogenic Activity in Sewage Treatment Works Effluents Was First Discovered

It was more than 25 years ago that sexual disruption in fish was first reported in fish in the River Lea catchment, Southeast England. Thames Water Authority staff, acting on casual observations by anglers, found a low incidence (around 5%) of intersex, or hermaphroditism, in populations of wild roach (*Rutilus rutilus*), a common lowland freshwater cyprinid, living in a sewage effluent settlement lagoon and in the river just downstream from a sewage treatment works (STW) effluent discharge (Sweeting, 1981). This finding was deemed very unusual because roach are gonochoristic (they have either a testis or an ovary), and hermaphrodites are believed to be very uncommon in this species (Jafri and Ensor, 1979; Schultz, 1996). The presence of intersex roach at the sites in question raised the possibility that compounds in STW effluent might cause disruption in sexual development. Quite independently, in the mid-1980s, while conducting studies on the endocrine control of reproduction in captive rainbow trout, our research, together with the U.K. Ministry of Agriculture, Fisheries, and Food (now called the Department of the Environment, Food, and Rural Affairs), discovered that the plasma of male fish contained measurable amounts of vitellogenin (VTG). VTG is normally synthesized by the liver in female oviparous (egg-laying) vertebrates in response to estrogen and is sequestered by developing

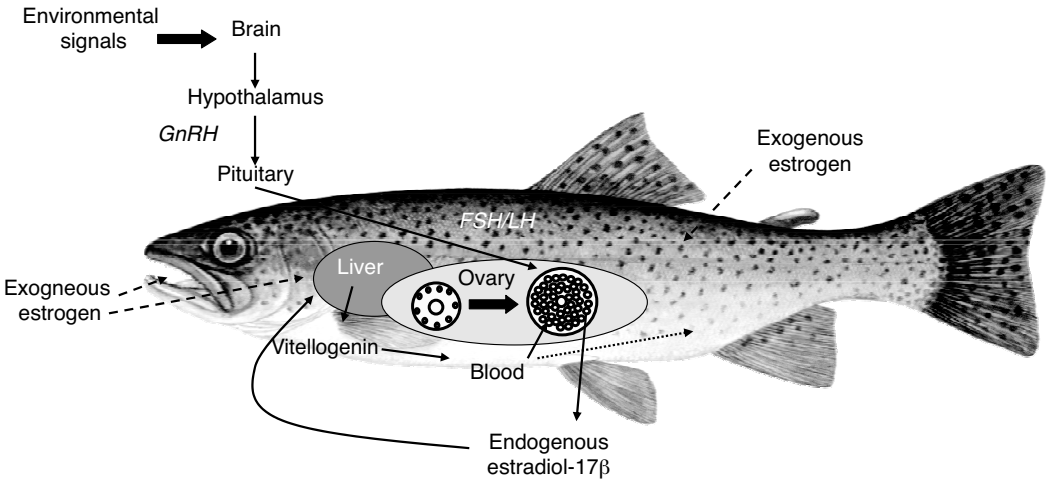

FIGURE 25.1 Vitellogenesis in fish. The solid line indicates the normal pathway of vitellogenin induction in female fish. Endogenous estrogen is produced by the ovary under the stimulation of FSH/LH; it passes into the circulation and induces vitellogenin synthesis in the liver. The vitellogenin then passes into the circulation and is sequestered by the developing oocytes and stored as yolk for the subsequent embryo. The dashed lines show the routes of exposure to exogenous estrogens in fish: oral and/or across the gill and/or skin surfaces. In male fish exposed to estrogen, the vitellogenin produced by the liver accumulates in the plasma (shown by the dotted line).

oocytes and stored as yolk to act as a nutrient reserve for the subsequent development of the embryo (Tyler and Sumpter, 1996) (Figure 25.1). The production of VTG, therefore, is usually restricted to females (Copeland et al., 1986; Tyler et al., 1996). Male fish, however, do contain the VTG gene, and exposure to both natural and synthetic estrogens can trigger its expression, resulting in detectable concentrations of VTG in the blood plasma (Chen, 1983; Sumpter and Jobling, 1995). The finding of VTG in male plasma indicated that an estrogen was present in the water, and the presence of STW effluent outfall upstream of the trout study site led to the hypothesis that the source of the estrogen was in the effluent. These initial observations of intersex wild roach and induction of VTG in male trout stimulated a series of investigations into the estrogenic properties of STW effluent.

In the late 1980s, a field study began in which caged rainbow trout were placed in effluents from sewage treatment works receiving either domestic inputs alone or both domestic and industrial inputs at 28 locations (covering all 10 Water Authority areas) throughout England and Wales (Purdom et al., 1994). The fish were exposed to the effluent for 2 to 3 weeks, after which time the plasma was assayed for VTG by radioimmunoassay (Sumpter, 1985). An additional five sites where the water supplies were uncontaminated with STW effluent were chosen as reference sites. At a few sites, the effluent was lethal to rainbow trout, but at the remaining sites where the fish survived the effluents were all found to be strongly estrogenic (Purdom et al., 1994). There was variability in the degree of the vitellogenic response in the fish deployed and it ranged from a 500-fold increase in plasma VTG up to over a 50,000-fold increase. At some sites, concentrations of plasma VTG exceeded 100 mg/mL; concentrations higher than those normally found in fully mature females (Tyler et al., 1990) (Figure 25.2). The variability in magnitude of the vitellogenic response between the fish at the different sites was probably the result of differences in the age and sex of the fish used at the different sites, the timing of fish deployment (exposures to effluents at the different sites were conducted at various times of the year), and the strength of the effluent to which the fish were exposed. Immature carp (*Cyprinus carpio*), a native U.K. cyprinid fish, were also exposed to the effluents at some of the test sites, and they produced a vitellogenic response similar to that in the trout (albeit less pronounced) (Purdom et al., 1994). These results clearly demonstrated that effluent from sewage treatment works throughout England and Wales were estrogenic.

The phenomenon of estrogenic effluent is not confined to the United Kingdom. Similar studies exposing fish to effluents from sewage treatment works, handling primarily domestic inputs, have been conducted in many other countries: Germany—bream (*Abramis brama*) (Hecker et al., 2001); France—chub

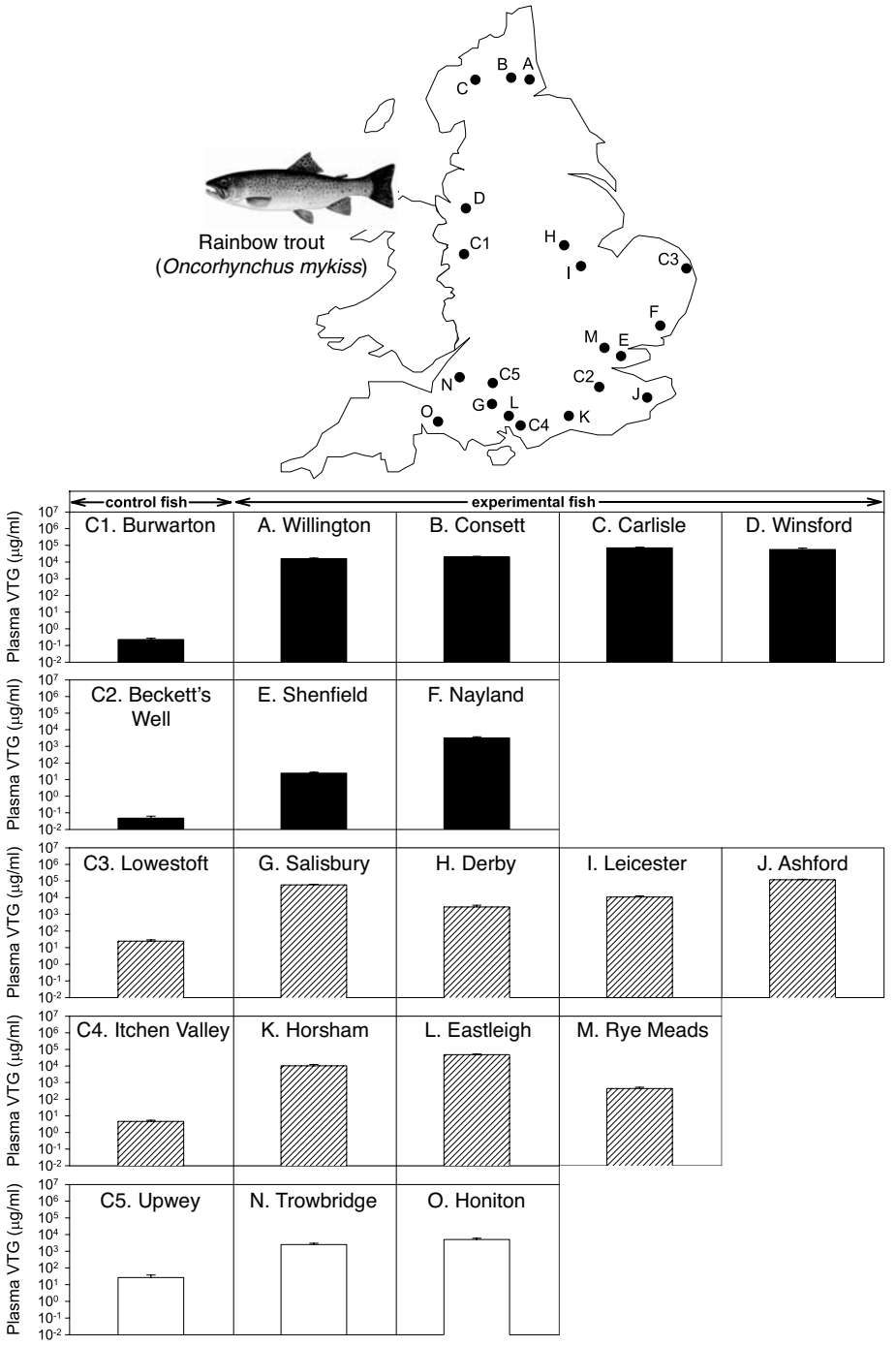

FIGURE 25.2 Induction of vitellogenin in rainbow trout (*Oncorhynchus mykiss*) placed in the effluent channel of 15 different sewage treatment works located throughout England. The fish were all males or immature females or mixed-sex immatures. Control fish were maintained at fish farms, which were supplied with high-quality water. The trout were maintained in the cages for between 2 and 3 weeks, and the plasma was assayed for vitellogenin. Solid bars represent male fish only, hatched bars represent immature females, and open bars represent immatures of mixed sex. Plasma vitellogenin concentrations are means ± SEM and are presented on a log scale. All fish held in effluent had elevated concentrations of plasma vitellogenin compared with their respective controls ($p < 0.001$). (Adapted from Purdom, C.E. et al., *Chem. Ecol.*, 8, 275–285, 1994.)

(Flammarion et al., 2000), roach (Minier et al., 2000), and eels (*Anguilla anguilla*) (R. Billard, pers. commun.); Sweden—rainbow trout (Larsson et al., 1999); Denmark—brown trout (*Salmo trutta*) (Bjerregaard et al., 2006); Portugal—common carp (*Cyprinus carpio*) (Diniz et al., 2005); Switzerland—brown trout (*Salmo trutta*) (Vermeirssen et al., 2005); and The Netherlands—bream (*Abramis brama*) (Vethaak et al., 2005). Studies exposing fish to effluents from sewage treatment works handling a mixture of both domestic and industrial influent have been carried out in the United States—common carp (Folmar et al., 1996); Norway—rainbow trout (Knudsen et al., 1997); The Netherlands—bream (Vethaak et al., 2005); United Kingdom—rainbow trout and common carp (Tyler et al., 2005); and China—medaka (*Oryzias latipes*) (Ma et al., 2005). In all of these studies, estrogenic responses (VTG induction) in exposed fish were demonstrated, although the magnitude of responses varied widely. The differences in the estrogenic potency of these effluent discharges probably depends on the influents received by the treatment works, the level and type of treatment that takes place in the sewage treatment works (Choi et al., 2006; Kirk et al., 2001; Liney et al., 2005), and the level of influent/effluent dilution.

A study on the Chelmsford sewage treatment work effluent in the United Kingdom showed that the estrogenic potency of this effluent varies on a seasonal basis; in the winter (November) a 1 in 2 dilution of the effluent induced a vitellogenic response in rainbow trout (exposed for 3 weeks), whereas in the summer (August) a 1 in 4 dilution resulted in VTG induction (Harries et al., 1999). Little seasonal variation occurs in the composition of the Chelmsford STW influent (it is primarily domestic in origin); therefore, the higher estrogenic potency of the effluent in the summer is likely to be a consequence of the lower level of dilution of the influent in the treatment works or of the effluent discharged. At this point in the discussion, it should be emphasized that the vitellogenic responses in caged fish that have been used to assess the estrogenic potency of STW effluent discharges in all of the studies mentioned above have been relatively short term (2 or 3 weeks in duration). In a study that exposed juvenile roach to the Chelmsford STW effluent, it was established that longer term exposures resulted in vitellogenic responses at lower effluent concentrations; an effluent concentration of $37.9 \pm 2.3\%$ induced a vitellogenic responses after 1 month, but only a $9.4 \pm 0.9\%$ effluent concentration did so after 4 months (Rodgers-Gray et al., 2000). This finding has important implications when assessing the possible impacts of treated sewage effluents in wild fish that can spend much, even most, of their lives exposed to these treated effluents in U.K. rivers.

Almost all treated sewage effluents studied in the United Kingdom have been found to be estrogenic, albeit to varying degrees, but this is not so in some other countries. In a study by Nichols and coworkers (1999) in central Michigan, no induction of plasma VTG occurred when caged fathead minnows (*Pimephales promelas*) were exposed to effluents from wastewater treatment plants handling both municipal and industrial influents. Similarly, common carp exposed to effluent from large sewage treatment works in Nevada did not show any consistent elevation in plasma VTG (Snyder et al., 2004). The reasons for the lack of an estrogenic response in fathead minnows at these sites in Michigan may relate to a higher efficiency of treatment of the influent or a greater dilution of the effluent compared with U.K. sewage treatment works. A report that documented the estrogenic activity of wastewater as it passed through five different sewage treatment works in the United Kingdom showed that where there was tertiary treatment more than 90% of the estrogenic activity of the influent was removed (Kirk et al., 2001). In a more recent study of the effluents from 17 sewage treatment works across Norway, Sweden, Finland, The Netherlands, Belgium, Germany, France, and Switzerland, it was also found that the nature of the treatment employed in each plant was an important factor determining its effectiveness at reducing the estrogenicity of the effluent (Johnson et al., 2005).

Estrogenic Activity in English Rivers

The widespread nature of estrogenic STW effluent in England and Wales prompted a series of investigations to determine whether the estrogenic activity in the STW effluent persisted in rivers downstream from point source discharges. Six rivers throughout England (namely, the Lea, Arun, and Kent Stour in southern England; the Chelmer and Essex Stour in eastern England; and the Aire in northern England) were studied at sites downstream of STW discharges (Harries et al., 1995, 1997) (Figure 25.3). The

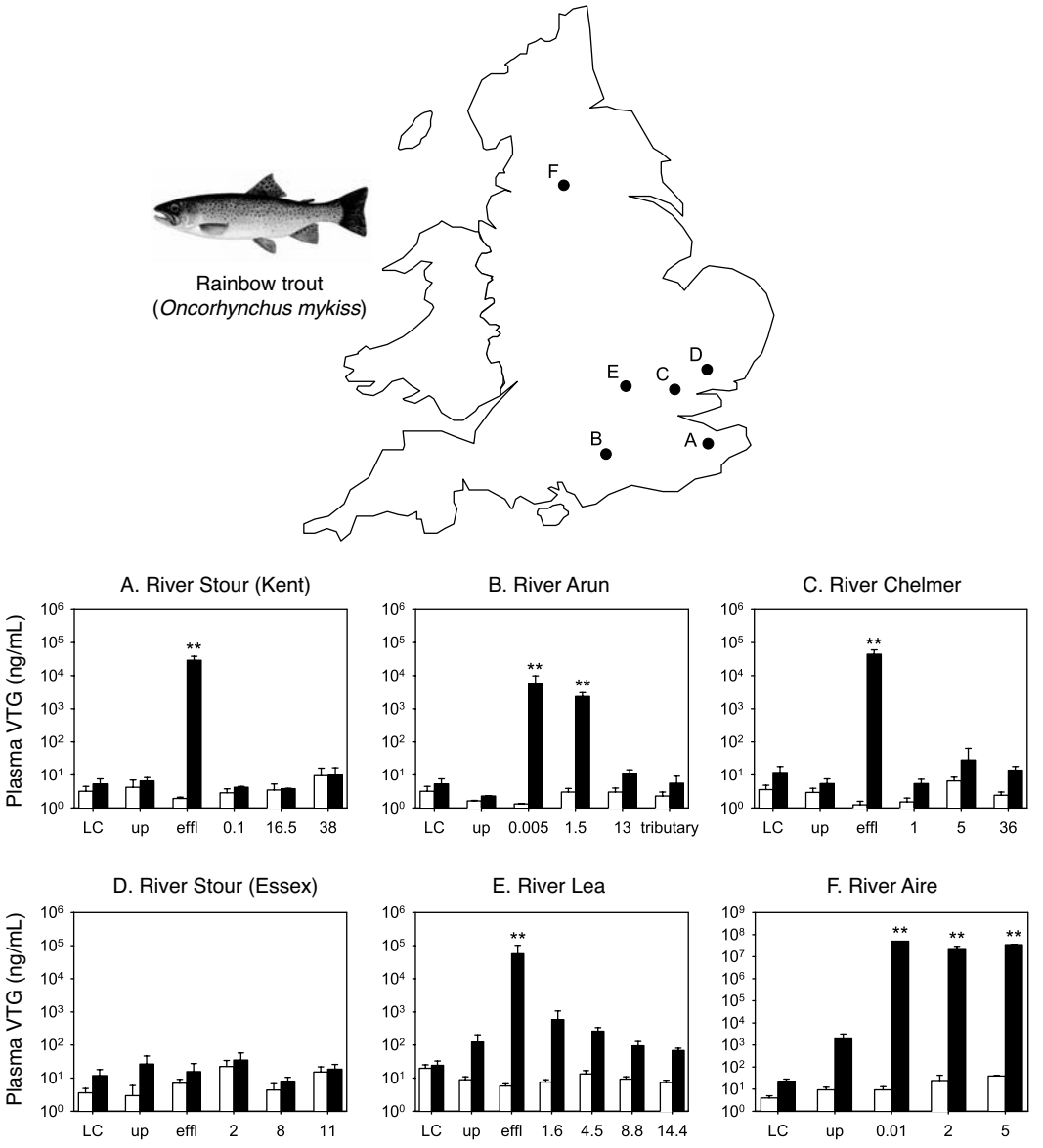

FIGURE 25.3 Induction of vitellogenin in caged male rainbow trout (*Oncorhynchus mykiss*) placed in effluent channels from sewage treatment works (effl.) and at various points upstream (up) and downstream of these discharges in 6 rivers in England. Distances downstream from the sewage treatment works are given in kilometers. Fish were exposed for 3 weeks, and the plasma was assayed for vitellogenin. Laboratory control (LC) fish were maintained for the same period of time in bore-hole water in the laboratory. Open and solid bars represent pretreatment and posttreatment plasma vitellogenin concentrations, respectively. Plasma vitellogenin concentrations are means ± SEM and are presented on a log scale. Significant differences between pre- and post-exposures are shown (**$p < 0.001$). (Adapted from Harries, J.E. et al., *Effects of Trace Organics on Fish: Phase 2*, Vol. FR/D 0022, report to the Department of the Environment Foundation for Water Research, Marlow, U.K., 1995; Harries, J.E. et al., *Environ. Toxicol. Chem.*, 16, 534–542, 1997.)

sewage treatment works in question handled mainly domestic effluent, but some (e.g., Marley STW, which discharges into the River Aire) also received a small (up to 7%) proportion of industrial influent. Adopting the same procedure as Purdom et al. (1994), caged rainbow trout were placed at various sites in the rivers for a period of 3 weeks, and the plasma was subsequently assayed for VTG. In the stretches of the rivers surveyed, no responses were seen in the Essex Stour, Kent Stour, or Chelmer (Harries et

al., 1997). In contrast, vitellogenic responses were seen in fish up to 1.5 km and 4.5 km downstream of discharges in the River Arun and River Lea, respectively (Harries et al., 1995, 1997) (Figure 25.3). Simultaneous measurements of testicular weight—gonadosomatic index (GSI) = gonad weight ÷ (body weight – gonad weight)—showed that the rate of testis growth was diminished at some, but not all, sites compared with the laboratory controls. The most marked effects seen at any of the sites studied were downstream of Marley STW on the River Aire in Yorkshire (Harries et al., 1997). This sewage treatment works treats influent from wool-scouring plants and the textile industry, as well as from domestic sources (see below). Significant elevations in concentrations of plasma VTG (that ranged between 25 and 52 mg/mL) were observed in the caged trout placed at all the sites along a 5-km stretch of the River Aire below the effluent discharge from Marley STW (Figure 25.3). Simultaneous measurements of the hepatosomatic index (HSI), which is equal to the liver weight ÷ (bodyweight – liver weight) and is an indicator of metabolic load/activity, showed that it was elevated in the fish at all the sites along the surveyed stretch of river. Furthermore, testis growth was significantly reduced in the trout at all study sites on the Aire. Subsequent to these findings with caged fish in the River Aire, tighter discharge limits and effluent treatment processes at source were introduced for the industries discharging alkylphenolic chemicals into this river through the Marley STW, and this action has resulted in a gradual reduction in the estrogenic effects seen (VTG induction) (Sheahan et al., 2002a,b). From the caged trout work, it was clear that the estrogenic activity in some rivers in the United Kingdom was not limited to areas immediately surrounding STW outfalls but rather persisted along significant stretches of rivers

Widespread Sexual Disruption in Wild Fish in English Rivers

Following the discovery of estrogenic activity in some U.K. rivers, a critical issue to address was whether the concentrations of estrogenic chemicals in the river were sufficient to exert adverse reproductive effects in wild fish. In fish, estrogens not only are responsible for VTG induction but also play important roles in sexual maturation (Bohemen and Lambert, 1981) and in sexual differentiation (Yeoh et al., 1996) and induce hepatic synthesis of vitelline envelope (eggshell) proteins (Hyllner et al., 1991). In fish, therefore, estrogens are vital for egg formation and provision of yolk for the developing embryo (Tyler et al., 1998). The main naturally occurring estrogens in fish (as in all classes of vertebrates) are estradiol-17β, estrone, and estriol. Both the amount of circulating endogenous natural estrogen and the timing of its release into the blood are carefully controlled by the hypothalamic–pituitary–gonadal axis. Inappropriate exposure to these estrogens (or their mimics) at critical times in the life cycle or at uncharacteristic concentrations may cause adverse effects (Hunter and Donaldson, 1983; Milston et al., 2003; Tyler et al., 1998). As an example, exposure to estrogens or estrogen mimics during the period of sexual differentiation has been shown to induce sex reversal and intersexuality by altering the normal developmental pathway of the reproductive ducts and the primordial germ cells such that they differentiate in a manner opposite to that of the genotypic sex of the individual (Balch and Metcalfe, 2006; Gimeno et al., 1996; Nash et al., 2004; van Aerle et al., 2002). Furthermore, exposure to estradiol-17β during sexual maturation has been shown to inhibit gonadal growth and development in male trout (Jobling et al., 1996). The possibility existed, therefore, that these reproductive effects might occur in wild populations of fish that were exposed to estrogenic substances in sewage effluents entering rivers.

In 1995, we undertook an extensive survey of wild roach in English rivers to determine whether sexual disruption was occurring in wild fish exposed to STW effluent. In this study, the presence of developing oocytes and/or an ovarian cavity in the testis of "male" roach was used as a diagnostic feature to characterize intersexuality in the captured fish (Jobling et al., 1998; Nolan et al., 2001) (Figure 25.4 and Figure 25.5). Roach of mixed age and sex were randomly sampled at locations upstream and downstream of sewage treatment works on eight rivers and at five reference sites throughout England and Ireland. The rivers selected for study varied with respect to the amount of sewage effluent they received, and, whenever possible, the sampling sites were selected where a physical barrier occurred (such as a weir) that separated the fish from the sites located upstream and downstream of the sewage treatment works. These barriers would limit the movement of fish between the two populations; fish downstream of the sewage treatment works could not migrate to the upstream population, although movement of fish from

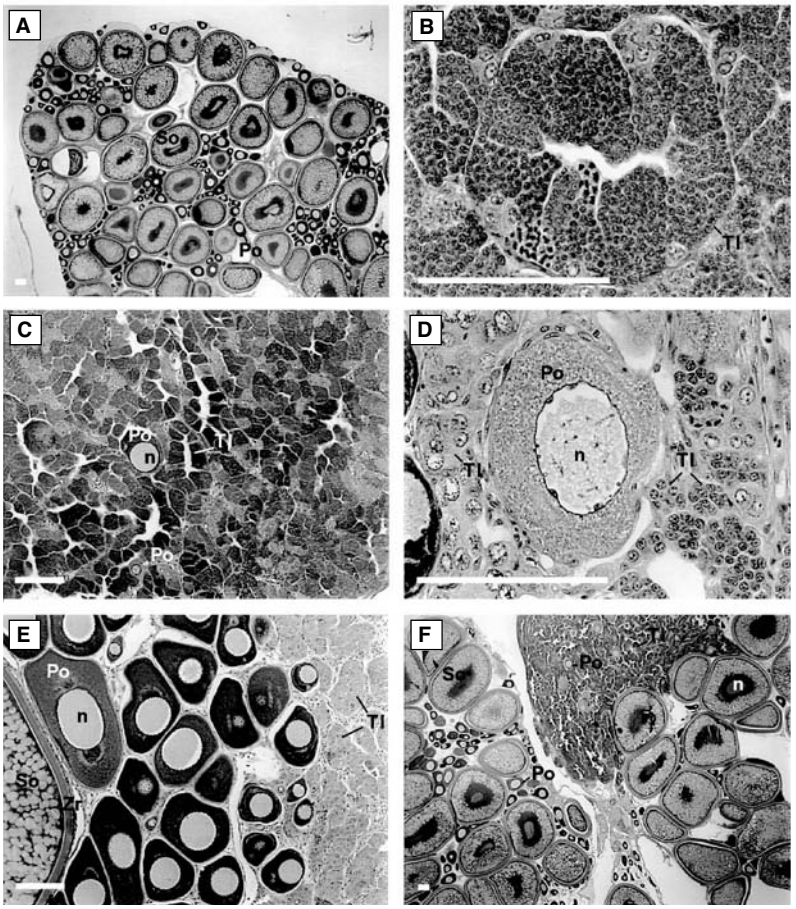

FIGURE 25.4 Histological sections showing the presence of oocytes in the testis of intersex roach (*Rutilus rutilus*). (A) Normal ovary; (B) normal testis; (C, D) mildly intersex gonad with both testicular germ cells and a few primary oocytes; (E, F) severely intersex gonad, with testicular germ cells and large numbers of both primary and secondary oocytes. Tl, testis lobule; Po, primary oocyte; So, secondary oocyte; Zr, zona radiata; n, nucleus. Scale bar represents 100 μm.

the upstream population to the one downstream may have occurred during periods of spate. Most of the reference sites were still waters (lakes and canals) because it proved extremely difficult to find rivers in the United Kingdom that contained healthy populations of roach but that did not receive effluent from sewage treatment works. Histological examination of the gonads revealed that a large proportion of the putative males were in fact intersex, as defined by the simultaneous presence of both testicular and ovarian characteristics (oocytes and/or an ovarian cavity) in the gonads. Intersex fish were found at all sites, but the incidence was considerably lower at the control sites (from 4% in the laboratory fish up to 18% at one field site) compared to that of populations of roach in rivers downstream of STW outfalls, where the proportion of intersex males ranged from 16% (River Wreake/Eye) to 100% (in both the River Aire in Yorkshire and the River Nene in Northamptonshire) (Jobling et al., 1998). In contrast, the incidence of intersexuality in roach at the upstream sites ranged from 11.7% (River Lea) up to 44.4% (River Nene), values that were typically lower than their respective downstream sites (Figure 25.6).

More recently, a considerably wider survey of roach living in U.K. rivers was carried out to further investigate for sexual disruption wild populations. In this work, 1615 roach were sampled from 51 river sites and feminization of the gonad and reproductive duct was found at 44 of those sites (86% of those studied) (Jobling et al., 2005). A more limited survey of juvenile roach living in 7 U.K. rivers with varying water quality found that the majority of roach from 5 of these rivers had female-like ducts (Beresford et al., 2004). The effects reported in wild fish in these English rivers are not limited to roach.

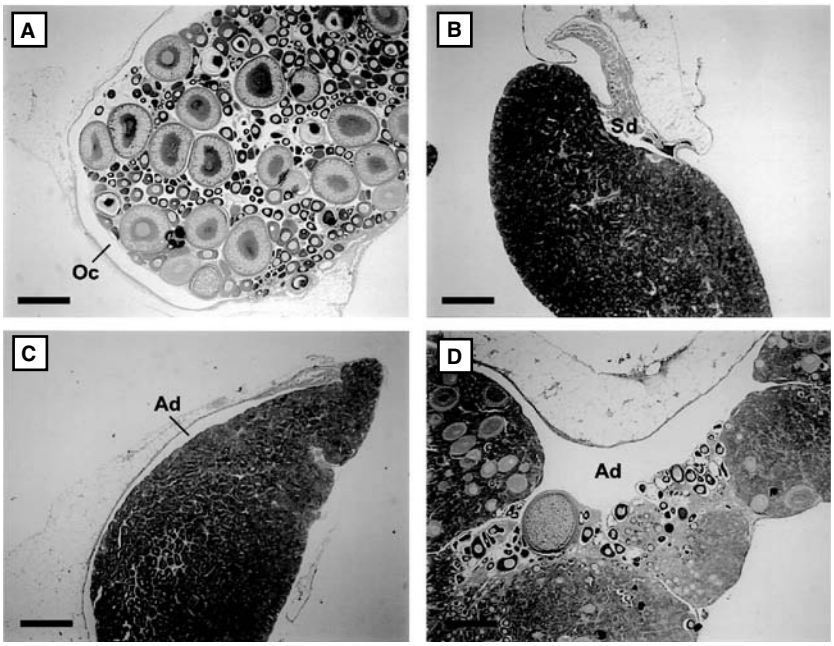

FIGURE 25.5 Histological sections showing the presence of aberrant ducts in the testis of intersex roach (*Rutilus rutilus*). (A) Ovary with normal ovarian cavity, which fully encapsulates the ovary; (B) testis with normal duct, which is derived from a clearly defined focal point; (C, D) intersex gonad with aberrant ducts. Oc, ovarian cavity; Sd, sperm duct; Ad, aberrant duct/ovarian cavity. Scale bar represents 500 μm.

A study on an allied cyprinid fish, the gudgeon (*Gobio gobio*), obtained from some of the same river sites showed a similar pattern of sexual disruption (van Aerle et al., 2001). The gudgeon is a cyprinid fish that has both a very different reproductive strategy and different ecological niche compared with the roach; the gudgeon is an asynchronous spawner (producing gametes at several times during the reproductive season) (Rinchard et al., 1993), and it lives on the bottom of the river, close to the river sediments, whereas the roach spawns only once during the reproductive season and lives mid-water in the river. Work on wild populations of stickleback (*Gasterosterous auculatus*) living in U.K. rivers also seems to provide evidence for estrogenic effects (P. Matthiessen, pers. commun.). Thus, reproductive disruption in fish in English rivers resulting from exposure to STW effluent does not appear to be species specific. In support of this, a number of *in vivo* and *in vitro* laboratory studies on estrogens and their mimics in a variety of animals have also indicated little species differences in specificity (Tyler et al., 1998), although species differences in sensitivity may occur (Routledge et al., 1998). Work on top predatory fish living in U.K. rivers found some evidence for estrogenic responses (vitellogenin induction) in pike (*Esox lucius*) and perch (*Perca fluvitalis*) living immediately downstream of discharges of treated sewage effluent, but very little evidence for any major disruption in gonadal sexual development (Vine et al., 2004). Whether this difference relates to differences in exposure regimes or differences in the physiology and sensitivities of these predatory fish to endocrine disruption remains to be determined.

In the studies on wild cyprinid fish, some individuals sampled from the control sites were also intersex, although the incidence and severity of the condition were very low compared with fish exposed to STW effluents. A low level of intersex may be a natural occurrence in wild cyprinid fish; alternatively, it may arise as a consequence of environmental disruption, such as exposure to estrogenic chemicals or possibly other endocrine-disrupting chemicals (EDCs) arising from non-point diffuse discharges sources, such as pesticides and herbicides from agricultural runoff. From our own (unpublished) studies on roach where we have bred roach in captivity in spring water, the incidence of natural intersex is less than 0.5%, suggesting that in the control fish in the field studies intersex has resulted predominantly as a consequence of environmental disruption. The degree (or severity) of intersexuality in the roach from

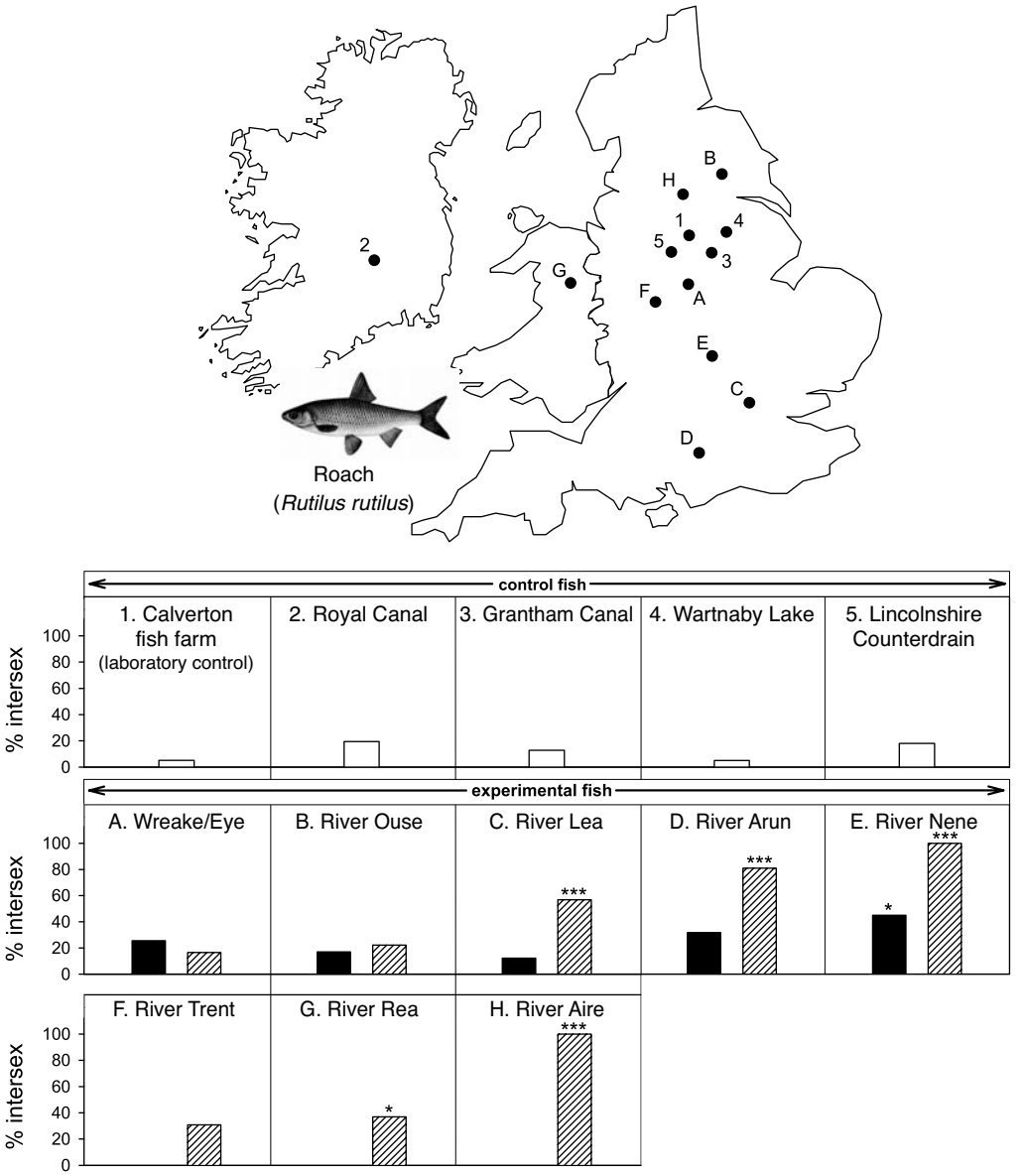

FIGURE 25.6 Incidence of intersexuality in male roach (*Rutilus rutilus*) from rivers and reference sites throughout England and Ireland. Percentage of intersex fish from river sites upstream and downstream of the sewage treatment works discharges are presented as solid and as cross-hatched bars, respectively. The percentages of intersex fish from reference sites are presented as open bars. Asterisks denotes significance from the field reference sites (*$p < 0.05$, ***$p < 0.001$). (Adapted from Jobling, S. et al., *Environ. Sci. Technol.*, 32, 2498–2506, 1998.)

the control sites was slight (often there were just a few primary oocytes in an otherwise normal testis), while in "male" fish from rivers receiving STW effluent the proportion of gonadal tissue that was ovarian sometimes exceeded 50%. In these individuals, the sperm duct was absent and was replaced by an ovarian cavity (Jobling et al., 1998; van Aerle et al., 2001) (Figure 25.5).

In the field studies on the roach and gudgeon, it was not possible to determine the genetic sex of the fish that were sampled because no sex-specific probes are available for these species or, indeed, for any other closely allied species. Evidence that the intersex fish were feminized genetic males was derived from the findings that (1) the number of roach with normal testes in any population was inversely proportional

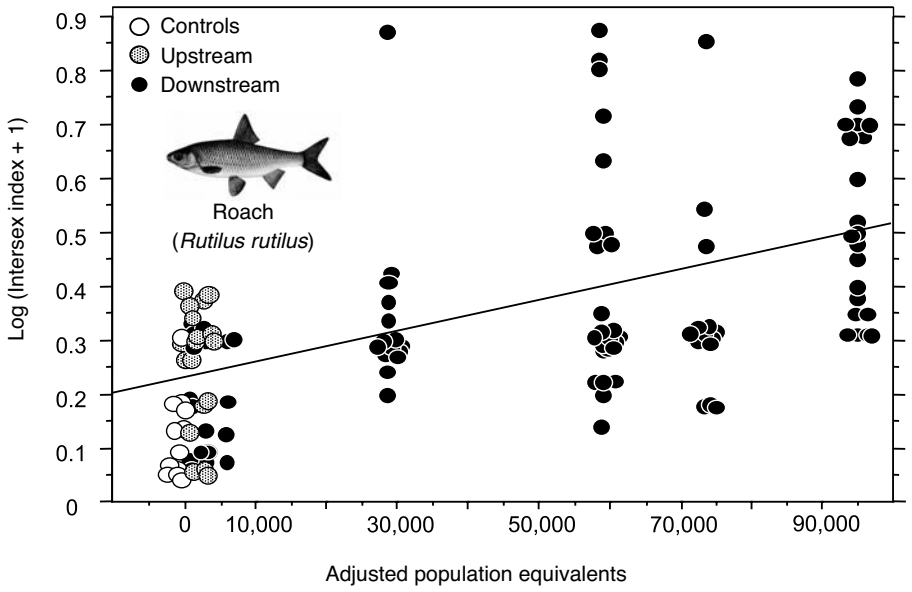

FIGURE 25.7 Correlation between intersex in roach (*Rutilus rutilus*) and the strength of effluent discharged into the rivers where the fish were caught. The degree of sexual disruption is presented as the logarithm of the intersex index, where the intersex index is a measure of the degree of sexual disruption (the degree of feminization of the testis—that is, the proportion of the testis occupied by oocytes and whether the gonad contains an ovarian cavity; see Jobling et al., 1998, for further details of the index). The concentration, or strength, of the effluent in the river at each sampling site was approximated by adjusting the population equivalent (a measure of the strength of the influent entering the sewage treatment works, where one population equivalent is the amount of biodegradable organic load that has a biochemical oxygen demand of 60 g of oxygen per day) to allow for the degree of dilution of the effluent in the river. The average dilution factor of the effluent in the river at the capture sites were calculated using hydrometric data of monthly river flows and actual sewage flows over a period of several years and for a time period encompassing the life spans of the fish captured wherever possible. Controls are fish sampled from waters that do not receive treated sewage effluent. Data points for upstream and downstream are fish sampled above and below major sewage treatment works outfalls, respectively. (Adapted from Jobling, S. et al., *Environ. Sci. Technol.*, 32, 2498–2506, 1998.)

to the number of intersex fish (Jobling et al., 1998); (2) sewage effluent discharges in the United Kingdom are predominantly estrogenic (Harries et al., 1997, 1999; Purdom et al., 1994; Rodgers-Gray et al., 2000, 2001) and anti-androgenic (see below); and (3) the male and intersex fish contained VTG in the plasma (Jobling et al., 1998; van Aerle et al., 2001), which provided strong evidence that some of the populations of wild roach were being exposed, and responding to, (feminizing) estrogenic contaminants. In the original field study on wild roach, there was a direct association (positive correlation) between the proportion of intersex fish and the concentration of the effluent at the different sampling sites (Jobling et al., 1998) (Figure 25.7). In summary, the results from the field studies on wild cyprinid fish in English rivers provided very strong evidence that it was the exposure to STW effluent (and the estrogenic chemicals therein) that caused the disruption in reproductive development (Jobling et al., 1998; van Aerle et al., 2001).

Mesocosm studies exposing roach to treated effluent from the Chelmsford and Great Billings STW have established that disruption in the development of the gonadal duct occurs as a consequence of exposure to treated sewage effluent during the period of sexual differentiation (Liney et al., 2005; Rodgers-Gray et al., 2001) (Figure 25.8). As yet, in our mesocosm studies we have not been able to induce altered sex cell development in fish. The reasons for this might be that the effluent used for the exposures did not contain a sufficient concentration of the causative agent or we have yet to expose the appropriate life stage or to expose the fish for long enough to cause this effect. Expanding on this, in some of our unpublished studies on wild roach living in U.K. rivers receiving treated sewage effluent we have shown a positive correlation between age of the fish (length of exposure) and the severity of the intersex condition. Furthermore, in an analysis of datasets from all of the wild roach caught over

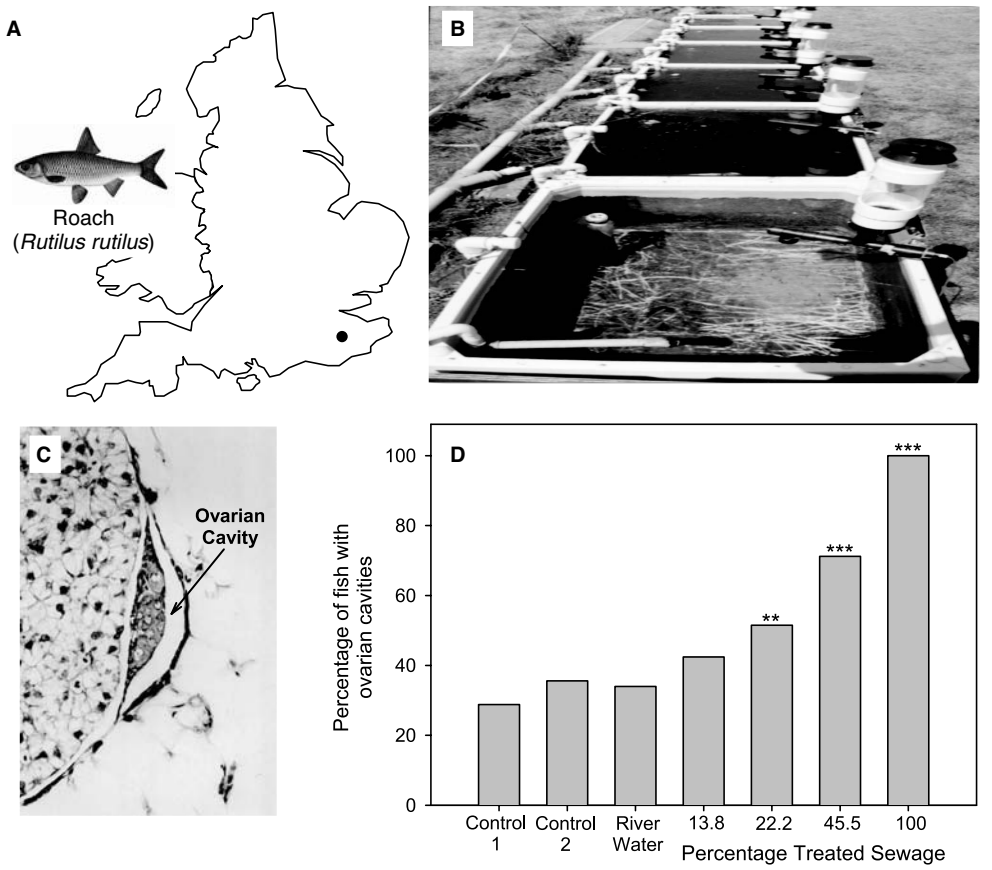

FIGURE 25.8 Feminization of the reproductive duct in juvenile roach (*Rutilus rutilus*) exposed to the Chelmsford sewage treatment works effluent. Fish were exposed to series of graded concentrations of treated sewage effluent for 150 days from 50 days post-hatch (dph), and effects on the sex ducts (presence of an ovarian cavity) were determined by histology. (A) Location of the Chelmsford sewage treatment works in the United Kingdom; (B) mesocosm used to expose juvenile roach to the Chelmsford effluent; (C) histological section of a male gonad (200 dph) with a feminized duct (ovarian cavity); (D) percentage of fish with an ovarian cavity exposed to different concentrations of treated sewage effluent. (Adapted from Rodgers-Gray, T.P. et al., *Environ. Sci. Technol.*, 35, 462–470, 2001.)

the past 10 years investigating for intersex has found that the intersex condition is seen only in fish that are over 2 years in age (S. Jobling, pers. commun.).

Studies on sexual disruption in wild fish on the European mainland have similarly identified intersex fish that also appear to have arisen as a consequence of exposure to effluents from sewage treatment works—for example, Germany, where Hecker et al. (2001) studied bream (*Abramis brama*); France (Minier et al., 2000); and Denmark (Christiansen et al., 2000). Sexual disruption in fish in the United Kingdom has also been demonstrated in coastal waters in the flounder (*Platichthys flesus*), with incidences of 17% in the Mersey estuary and 7% in the Tyne estuary, but it has not been determined if this is as a consequence of exposure to STW effluents (Allen et al., 1999; Kirby et al., 2004; Matthiessen, 2003). Differences in the incidence and severity of intersex in wild fish living in contaminated waters appear to vary widely, and they are likely to depend not only on their level of exposure to EDCs (determined, at least in part, by the organism's ecological niche) but also on differences between species in their sensitivity to the chemicals in question, an issue that has, as yet, received very limited attention.

The findings from wild fish from English rivers strongly support the hypothesis that the concentration of sewage effluent in a river is a major causal factor in the evolution of intersexuality. Furthermore, as the majority of the sampling sites in the field studies on roach and gudgeon were several kilometers downstream

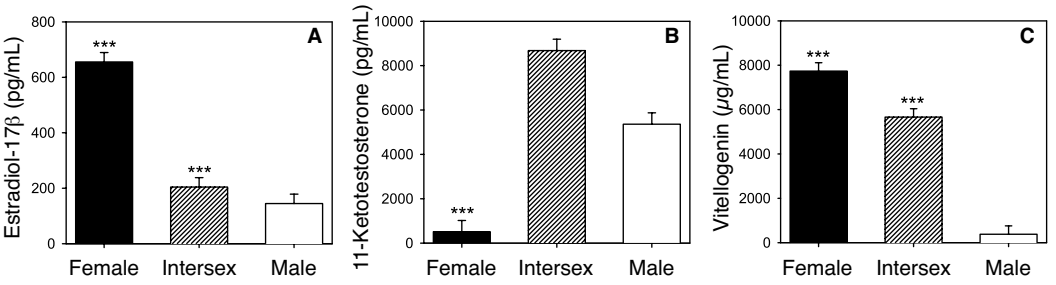

FIGURE 25.9 Endocrine status of roach (*Rutilus rutilus*) sampled from wild populations in the United Kingdom. Fish from effluent contaminated waters were collected from the River Nene (Northamptonshire) and Aire (Yorkshire). Roach from sites uncontaminated with treated sewage effluent were sampled from the Royal Canal, Ireland, Grantham Canal, Leicestershire, and a spring-fed Lake at Wartnarby, Leicestershire. Fish were collected in October. (A) Plasma estradiol-17β, (B) 11-ketotestosterone, and (C) vitellogenin in male, female, and intersex fish. Asterisks represent significant differences of intersex male or exposed females from pooled control males or females, respectively (***$p < 0.001$). (Adapted from Jobling, S. et al., *Biol. Reprod.*, 66, 272–281, 2002.)

from any point of sewage input, it is likely that the fish collected were truly representative of populations in typical English river ecosystems. The ecological implications of intersexuality will depend on whether the reproductive competence of the fish that are intersex is compromised. Our studies have shown that wild intersex roach (*Rutlius rutilus*) and male roach living in effluent contaminated rivers in England have altered sex steroid hormone profiles (Figure 25.9), altered spawning times, and reduced sperm production (Jobling et al., 2002a). When all data for male, intersex, and female fish were pooled across all the sampling sites, the plasma concentrations of estradiol-17β and vitellogenin in the intersex fish were intermediate between the concentrations found in male and female fish. Female fish had considerably lower 11-ketotestosterone concentrations than in either male or intersex fish and there were no significant differences in the concentration of 11-ketotestosterone between the male and intersex fish (Jobling et al., 2002a).

Examining the reproductive potential of intersex fish, we have also established that the ability of intersex roach to produce gametes is highly variable and is dependent on the degree of disruption in the reproductive ducts or altered germ cell development. Small numbers of wild roach have been found that cannot produce any gametes at all due to the presence of severely disrupted gonadal ducts. In the majority of intersex fish found, male gametes are produced that, although viable, are of poorer quality than those from males obtained from aquatic environments that do not receive treated sewage effluent (Jobling et al., 2002b). Fertilization and hatchability studies have shown that intersex roach, even with a low level of gonadal disruption (mildly intersex), are compromised in their reproductive capacity and produce fewer offspring than fish from uncontaminated sites under laboratory conditions (Jobling et al., 2002b). In that study, there was an inverse correlation between reproductive performance (defined by the ability to produce viable offspring) and severity of gonadal intersex (Figure 25.10). This, in turn suggests that the intersex condition is likely to have population level consequences. This question is of fundamental importance for the sustainability of wild populations of fish.

Identification of Estrogenic Substances in STW Effluent and Evidence That They Cause Feminization of Fish in English Rivers

Sewage treatment works that receive domestic, industrial, and agricultural waste release a complex (and ill-defined) mixture of natural and synthetic chemicals into the aquatic environment following their partial or complete biodegradation during the treatment process. The scale of the problem of isolating causal compounds responsible for biological effects in aquatic wildlife can be gauged from reported estimates that over 60,000 manmade chemicals are in routine use worldwide (a total of over 100,000 manmade chemicals are discharged into the environment), and anywhere between 200 and 1000 new synthetic chemicals enter the market each year (Shane, 1994); thus, identifying specific chemicals

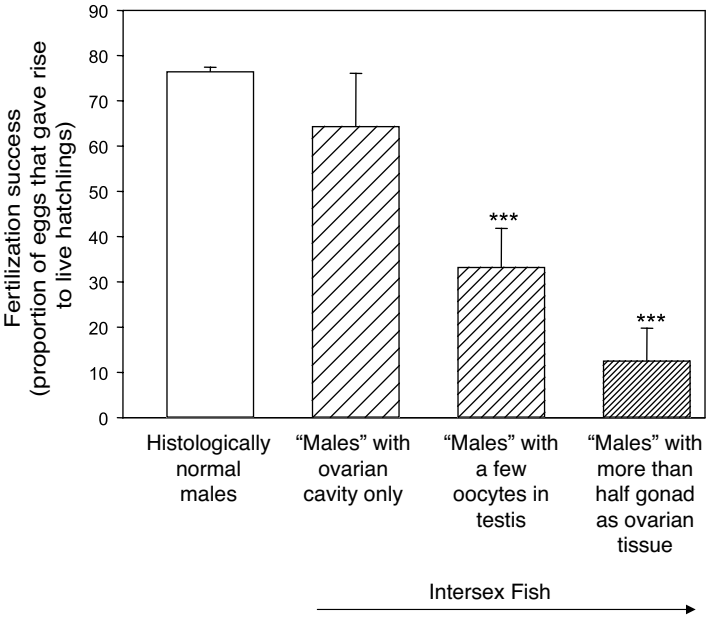

FIGURE 25.10 Relationship between the degree of sexual disruption and reproductive performance (proportion of eggs that gave rise to live hatchlings) in wild roach (*Rutilus rutilus*) sampled from the River Arun. Intersex fish were grouped into three classes according to the degree of severity of the intersex condition: feminized ducts only, gonads containing some oocytes, and 50% or more of the gonad female. Asterisks represent significant differences between intersex fish and males (***$p < 0.001$).

responsible for adverse effects observed in the field is difficult. Testimony to this is the fact that there are very few examples where an adverse effect in any aquatic wildlife species has been conclusively linked to a specific chemical. In the United Kingdom, the search for the causative agents in STW effluents and rivers responsible for VTG induction and feminization of male fish (intersexuality) has focused on estrogens because of the estrogen-dependent nature of the responses seen. Effluents, however, may potentially contain a wide variety of estrogens, including steroidal estrogens, phytoestrogens, mycoestrogens, and manmade estrogen mimics. Indeed, it has now been established that a wide variety of synthetic chemicals are capable of mimicking estrogens, and they are structurally diverse. These chemicals include alkylphenolic chemicals, many of which result from the breakdown of non-ionic surfactants (Jobling and Sumpter, 1993; Nimrod and Benson, 1996); plasticizers, such as bisphenol-A (Brotons et al., 1995; Krishnan et al., 1993; Sohoni et al., 2001) and some phthalates (Jobling et al., 1995; Harries et al., 1997); and certain pesticides and herbicides and their products of metabolism and environmental degradation (Soto et al., 1995; Tyler et al., 2000), many of which are likely to enter sewage treatment works. Most of these estrogen mimics are weakly biologically active compared with steroidal estrogens (Tyler et al., 1998) and may only cause endocrine modulation in wildlife if: (1) they circulate in the environment at high concentrations, (2) their breakdown is slow or they bioaccumulate, or (3) they are in widespread regular use and are entering the environment almost constantly. All of these criteria, however, are true for certain manmade chemicals, such as alkylphenols (see below).

A toxicity identification evaluation (TIE) approach has been used successfully to identify the causative agents in English STW effluents for some of the estrogenic effects in fish (Desbrow et al., 1998; Rodgers-Gray et al., 2000, 2001). In the work of Desbrow and colleagues, effluent from seven English sewage treatment works receiving primarily domestic inputs were analyzed for estrogenic contaminants: South-End-on Sea, where the effluent had only primary treatment before being discharged into the marine environment; Harpenden, Rye Meads, and Deephams sewage treatment works in the catchment of the River Lea; Horsham sewage treatment works, which discharges into the River Arun (all of these effluents were known previously to be estrogenic); Billing sewage treatment works, which discharges into the

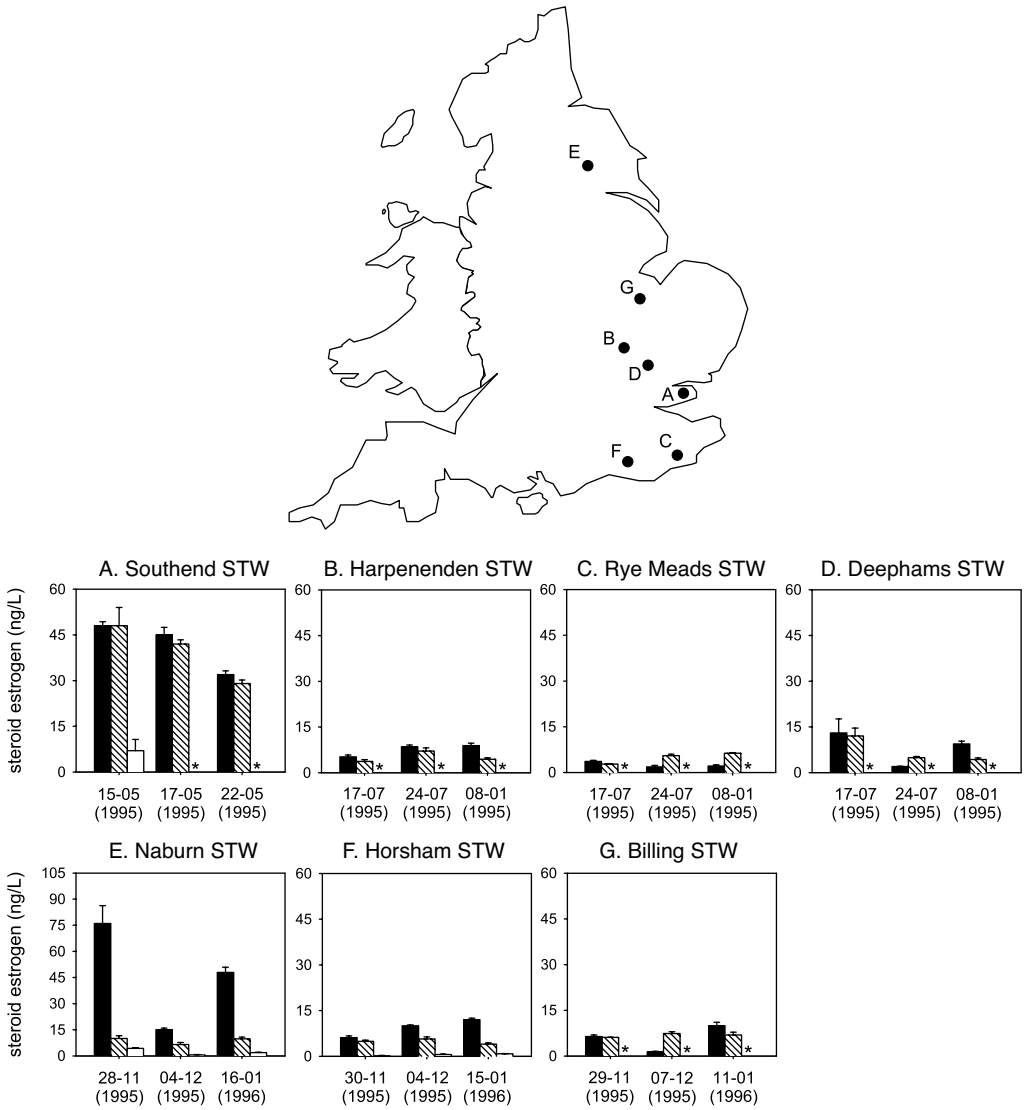

FIGURE 25.11 Concentrations of natural and synthetic steroid estrogens detected in seven effluents (receiving primarily a domestic influent) discharging into British waters. Solid bars represent estrone; hatched bars, estradiol-17β; and open bars, ethinylestradiol. Values represent mean and standard deviation of three replicate injections of the sample into the GC–MS. Three separate samples were collected for each site (dates are given). *Steroid was nondetectable. (Adapted from Desbrow, C. et al., *Environ. Sci. Technol.*, 32, 1549–1558, 1998.)

River Nene; and Naburn sewage treatment works, which discharges into the River Ouse. Effluent from Marley sewage treatment works on the River Aire, which receives industrial input from the wool-scouring industry, was also analyzed (see Figure 25.11). Effluents were chemically fractionated into samples of decreasing complexity, which were then assessed for estrogenicity using an estrogen-responsive yeast cell line. When the yeast (which in this case expressed the human estrogen receptor) comes into contact with an estrogenic chemical, an enzyme is produced that mediates a color change in a chromogenic substance in the medium, and this activity can be measured colorimetrically (Routledge et al., 1996). By adopting this approach it was quickly established that the estrogenic components in the effluent were largely confined to the dissolved phase (Desbrow et al., 1998). The estrogenic compounds were subsequently

subfractionated on C-18 columns, separated by high-performance liquid chromatography (HPLC), and identified using gas chromatography–mass spectrometry (GC–MS). Despite the complex composition of the domestic effluents tested, only a small number of estrogenic compounds were identified—namely, the synthetic estrogen ethinylestradiol and the natural steroidal estrogens estradiol-17β and estrone (Desbrow et al., 1998). Steroid estrogens have also been identified as the major estrogenic contaminants in many other U.K. treated sewage effluents (Liney et al., 2005; Rodgers-Gray et al., 2000, 2001). Alkylphenolic chemicals (resulting from the biodegradation of alkylphenol polyethoxylate surfactants) were identified as another major source of estrogenicity in the effluent from the Marley sewage treatment works (Waldock et al., 1997), in effluents discharging into the River Mersey and Tees (Blackburn et al., 1999), and as more minor estrogenic contaminants in the Chelmsford STW effluent (Liney et al., 2005; Rodgers-Gray et al., 2000, 2001).

Natural and Synthetic Steroidal Estrogens

Concentrations in Effluents and Rivers

Analysis of the English effluents by Desbrow et al. (1998) indicated that most of the estrogenic activity (>80% total activity) in domestic effluent occurred in a single fraction, which contained low levels of natural and synthetic steroidal estrogens. Measurements based on 3 separate sampling intervals indicated that estradiol-17β was present at mean concentrations ranging between 4.8 ± 1.1 μg/L (Rye Meads STW) and 39.7 ± 5.6 μg/L (Southend STW), and estrone was present at concentrations between 5.9 ± 2.4 μg/L (Billing STW) and 46 ± 17.7 μg/L (Naburn STW) (Figure 25.11). Studies on the Chelmsford effluent in the United Kingdom have recorded concentrations of estradiol-17β between 4 and 88 μg/L and estrone at concentrations ranging between 15 and 220 μg/L (Liney et al., 2005; Rodgers-Gray et al., 2000). The presence of steroidal estrogens in effluents is not restricted to the United Kingdom; for example, in the Tel Aviv area (Israel), natural steroidal estrogens have been detected in raw sewage at concentrations ranging from 48 μg/L up to 141 μg/L (depending on drought conditions). Moreover, estrogen levels in treated sewage water (used for irrigation) discharged from small farm-based sewage treatment units and municipal sewage treatment works in Israel have been measured at concentrations between 39 and 153 μg/L, with concentrations that are 2- to 3-fold higher in the summer months (Shore, 1993). In Germany, estradiol-17β levels in STW effluent have been reported at concentrations of up to 20 μg/L (Stumpf et al., 1996). In a study in Sweden, estradiol-17β and estrone were measured in a STW effluent at concentrations of 1.1 μg/L and 5.8 μg/L, respectively (Larsson et al., 1999).

The mean concentration of ethinylestradiol in effluents from the study on the 7 different English sewage treatment works (based on 3 separate sampling intervals) ranged from nondetectable in 4 (Harpenden, Rye Meads, Deephams, and Billing) to between 0.53 ± 0.18 μg/L (Horsham) and 7.0 ± 3.7 μg/L (Southend, based on only one sample time point) (Figure 25.11). In the Chelmsford STW effluent, ethinylestradiol concentrations ranged from nondetectable (<0.5 μg/L) to 1.4 μg/L (Gibson et al., 2005; Liney et al., 2005; Rodgers-Gray et al., 2001). In Sweden and Germany, concentrations of ethinylestradiol in sewage treatment works have been recorded at concentrations up to 4.5 μg/L (Larsson et al., 1999) and 62 μg/L (Stumpf et al., 1996), respectively. Concentrations of ethinylestradiol in English rivers have been reported as high as 15 μg/L (measured using a radioimmunoassay procedure) (Aherne and Briggs, 1989), but generally they have been found at far lower concentrations (undetectable to very low micrograms-per-liter concentrations). Concentrations of ethinylestradiol in river water in other European countries have been recorded up to 5 μg/L, but again generally at much lower concentrations (less than 0.5 μg/L) (Cargouet et al., 2004; Stumpf et al., 1996; Vethaak et al., 2005).

Some of the differences in the concentration of steroidal estrogens reported in STW effluent and rivers in the different studies may, at least in part, be a function of the steroidal extraction procedures employed or whether or not deconjugation of the steroids takes place (see below). In addition, differences in the level of treatment that occurs during the STW process, the concentration of the influent entering the STW plant, the level of bacterial activity (affecting the rate of degradation of estradiol-17β to estrone), and the extent to which the STW effluent is diluted in the river following discharge will also affect

steroid concentrations in the STW effluent and river. Some of these factors vary depending on the time of year. A study on the Chelmsford STW effluent measured concentrations of estrone and estradiol-17β in the summer (August) of up to 56 μg/L and 8.8 μg/L, respectively, whereas in the winter (November) they were up to 220 μg/L (estrone) and 88 μg/L (estradiol-17β), respectively, based on composite samples collected over a 5-day period (Rodgers-Gray et al., 2000).

Although the reported concentrations of steroidal estrogens in STW effluents and river waters in the different studies have been variable, it is clear that their presence in the aquatic environment is a common European phenomenon. In certain countries, such as Israel, the primary sources of steroidal estrogens detected in the effluent were probably agricultural in origin (Shore, 1993). The effluents analyzed in the English study (with the exception of Marley STW), however, received little or no agricultural input (they came from sewage treatment works located in urban areas); therefore, the steroidal estrogens detected are believed to be human in origin rather than a consequence of intensive animal farming practices (Desbrow et al., 1998). The primary source of these natural steroidal estrogens in the English STW effluents is likely to be of human origin. To illustrate this, women excrete between 10 and 100 μg of estrogen daily, depending on the phase of their menstrual cycle (Aldercreutz et al., 1986). Pregnant women may secrete up to 30 mg of estrogen a day. In the United Kingdom, the major source of ethinylestradiol in effluent is probably derived from the contraceptive pill. In women, most of the estrogen (both natural and synthetic) is excreted, predominantly in the urine, as inactive glucuronated and sulfated conjugates (Kulkarni and Goldzieher, 1970; Maggs et al., 1983). The natural and synthetic steroids detected in the effluent from English sewage treatment works, however, were found to be in an unconjugated (biologically active) form, rather than the polar (water-soluble) glucuronide or sulfated metabolites. It is likely, therefore, that the inactive conjugated natural and synthetic steroidal estrogens, eliminated from the body, are deconjugated during the sewage-treatment process into biologically active parent compounds, which are then subsequently discharged into the rivers. It has been shown that certain microorganisms (such as *Escherichia coli*) present in sewage sludge that produce the enzyme β-glucuronidase (Dray et al., 1972) can effectively transform glucuronated estradiol, which is not estrogenic, into a form capable of stimulating an estrogenic response (VTG production) in fish exposed via the water (Panter et al., 1998).

Estrogenic Activity in Fish

Natural steroidal estrogens are very potent hormones and have biological activity at very low concentrations in fish and in other animals, and laboratory studies have shown that the concentrations of the estradiol-17β and estrone present in STW effluent in England are sufficient to explain the induction of VTG synthesis in caged fish placed close to effluent discharges. Threshold concentrations for a vitellogenic response to estradiol-17β in rainbow trout range between 4.7 μg/L in juvenile females exposed for 14 days (Thorpe et al., 2001, 2003) and 10 μg/L in adult males exposed for 3 weeks (Routledge et al., 1998). Threshold concentrations for a vitellogenic response to estradiol-17β in cyprinid fish are a little higher, at between 50 and 100 μg/L for mixed-sex adult roach exposed for 3 weeks (Routledge et al., 1998) and early-life-stage fathead minnows exposed as embryos from 24 hours after fertilization through to hatch and up to 30 days after hatch (Tyler et al., 1999). The threshold concentration for a vitellogenic response for estrone in both salmonid and cyprinid fish appears to be similar, between 25 and 50 μg/L (Panter et al., 1998; Routledge et al., 1998). Exposure of fish to concentrations of estradiol-17β of 100 μg/L and higher, even for relatively short periods of time (21 days), can also inhibit testicular growth, cause deleterious effects on spermatogenesis, and even cause complete regression of the testes (Halm et al., 2001; Kinnberg et al., 2000; Panter et al., 1998). Induction of intersex and even complete sex reversal can also be induced by exposure to higher concentrations of natural steroid estrogens (Baroiller et al., 1999; Hahlbeck et al., 2004; Iwamatsu, 1999; Nimrod and Benson, 1996). Exposure of zebrafish to estradiol-17β (5, 25, and 100 μg/L) for 3 weeks prior to (from fertilization to 21 days post-fertilization [dpf]) and during the time of sex differentiation (from 21 to 42 dpf) resulted in a dose-related formation of an ovarian-like cavity in presumptive males. In fish exposed to 100 ng estradiol-17β per L from 21 to 42 dpf, alterations in germ-cell development (oocytes in the testis) and a significant change of the sex ratio toward the female sex were observed (Brion et al., 2004).

It is worth emphasizing that care has to be taken when ascribing species sensitivities to natural (and synthetic) estrogens, because the stage of the reproductive cycle at the time of exposure may affect the magnitude of the response (Billard et al., 1981). The effects of estradiol-17β in the medaka can illustrate this. Iwamatsu and coworkers (1999) found that exposure of medaka early life stages to 1 μg/L estradiol-17β (for 20 days from fertilization to 10 days post-hatch [dph]) induced gonadal feminization, but ten times this amount of estradiol-17β was required to cause sex reversal in adult medaka. Together, these laboratories studies strongly support the contention that natural steroid estrogens present in STW effluents are contributing to the altered patterns of gonadal development seen in wild fish in U.K. rivers. High concentrations of estradiol-17β (1 to 10 μg/L) have also been shown to inhibit both sexual behavior and the development of secondary sexual characteristics in fish, including goldfish (*Carassius auratus*) (Bjerselius et al., 2001) and guppy (*Poecilia reticulata*) (Bayley et al., 1999), but whether the lower levels of steroidal estrogens present in English rivers have discernible effects on fish behavior and secondary sexual characteristics has not been studied and indeed will be more difficult to quantify.

Ethinylestradiol is the most potent inducer of vitellogenesis reported to date, and exposure of male rainbow trout to concentrations in the water of only 0.1 μg/L and above causes a rapid and pronounced synthesis of VTG (3-week exposures) (Purdom et al., 1994; Sheahan et al., 1994). In male adult fathead minnow exposed to ethinylestradiol for 3 weeks, the least effective concentration for VTG induction was similarly less than 1 μg/L (our own unpublished data). The potency of ethinylestradiol exceeds that of natural steroid estrogens *in vivo*. For VTG induction in fish, ethinylestradiol is between 20 and 50 times more potent than estradiol-17β (Thorpe et al., 2001, 2003), and for induction of intersex it is up to 100-fold more potent (Metcalfe et al., 2001). The greater potency of ethinylestradiol compared with natural steroid estrogens is likely to be due to the 17α-ethinyl group that increases its longevity by reducing the rate of metabolism at C-16 and C-17 (Guengerich, 1990) and its capacity to bioaccumulate (10,000 times in bile in rainbow trout after only a 3-week exposure) (Larsson et al., 1999). In the rainbow trout, maximal rates of VTG synthesis have been shown to occur at concentrations of ethinylestradiol at, or below, a concentration of 10 μg/L (Purdom et al., 1994; Thorpe et al., 2001, 2003).

Less is known about the reproductive consequences of exposure of oviparous animals to ethinylestradiol; however, it is known that a single dose of 2 μg/L ethinylestradiol in water can significantly retard the growth and development of the testes in maturing male trout (Jobling et al., 1996). In a study on the fathead minnow, exposure to 10 μg/L ethinylestradiol for only a brief period during the period of sexual differentiation (10 to 15 dph) has been shown to result in feminization of the gonadal duct (formation of an ovarian cavity) in 60% of the male population (Figure 25.12) (van Aerle et al., 2002). Furthermore, exposure of mature fathead minnows to ethinylestradiol at a concentration of 10 μg/L for a period of 4 weeks resulted in a reduction in the number of eggs oviposited (Lange et al., 2001). Recent studies have shown that exposure to low microgram-per-liter concentrations of ethinyloestradiol impact negatively on reproductive output in zebrafish (Hill and Janz, 2003). Importantly, exposure of zebrafish for long periods to only 5 μg/L has been shown to result in reproductive failure in the population (Nash et al., 2004). Thus, although the concentrations of ethinylestradiol in English rivers are likely to be very low (low micrograms per liter or less) (Desbrow et al., 1998; Rodgers-Gray et al., 2001), the extreme potency of this synthetic estrogen, together with its capacity to bioaccumulate, means that, even at these concentrations, it could be a major contributor to the estrogenic responses observed in fish exposed to domestic effluents in U.K. rivers.

Alkylphenolic Chemicals

Concentrations in Effluents and Rivers

Alkylphenol polyethoxylates (APEOs) are nonionic surfactants that have been used for over 40 years, primarily in the manufacture of plastics, elastomers, agricultural chemicals, paper (pulping and deinking), and industrial detergent formulations. APEOs are one of the largest volume surfactants in

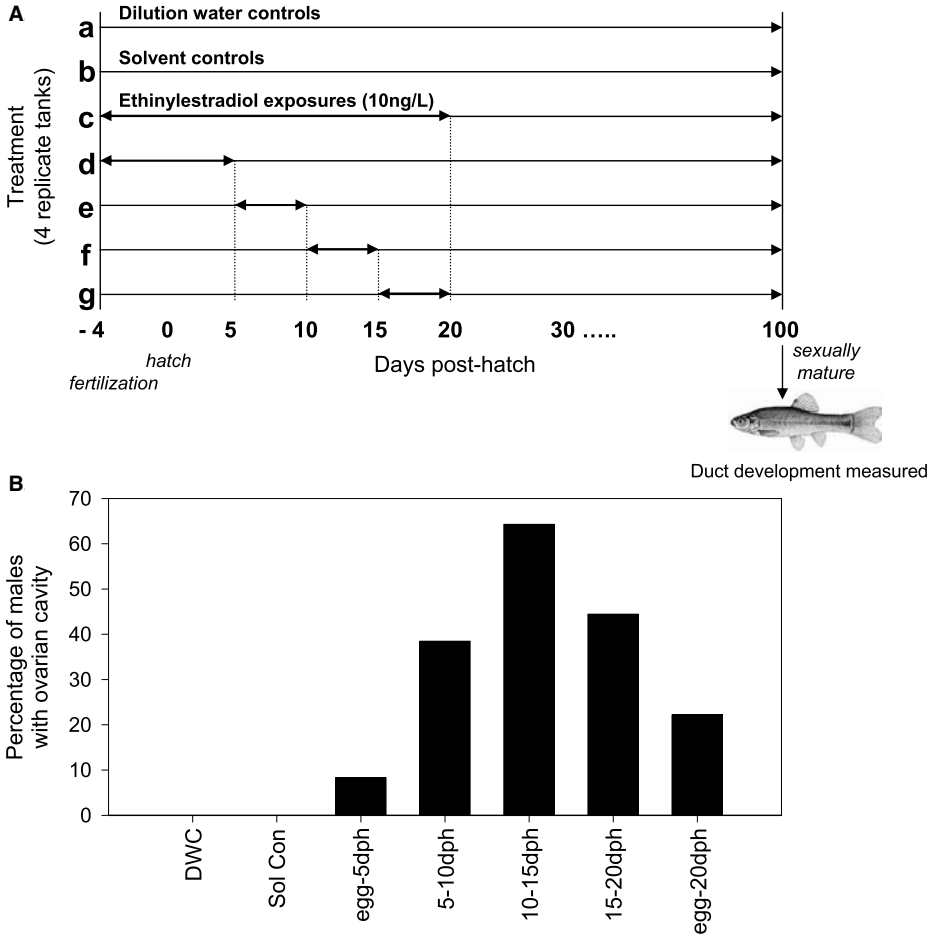

FIGURE 25.12 Window of sensitivity for gonadal duct disruption in fathead minnows (*Pimephales promelas*) exposed to ethinylestradiol during their early life stages. (A) Ethinylestradiol exposure regimes; (B) proportion of male fish with feminized ducts (ovarian cavity) in fish at 100 days post-hatch. (Adapted from van Aerle, R. et al., *Ecotoxicology*, 11, 423–434, 2002.)

production (CMA, 1991); in the United States, they comprise 6% of the total surfactant production and 25% of the total nonionic surfactant production (Nimrod and Benson, 1996). In 1988, over 360,000 tons of APEO surfactants were produced worldwide (Ahel et al., 1993), of which nonylphenol ethoxylates (NPEOs) and octylphenol ethoxylates (OPEOs) were the most common, constituting 82% and 15% of the market production, respectively (Naylor, 1992). It is estimated that around 60% of the 114 million kilos of APEOs used every year in the United States enters the aquatic environment following their disposal in wastewater (Naylor, 1992). In a report, commissioned by the United Kingdom's Department of the Environment, it was estimated that 83% of the U.K. NPEO production ends up in the environment, with 37% in the aquatic environment (CES, 1993). Nearly all of this receives biological treatment before being discharged into rivers and other water bodies. Some domestic sewage effluents can contain up to hundreds of micrograms of alkylphenolic compounds per liter (Blackburn and Waldock, 1995). Alkylphenols (APs), such as nonylphenol (NP) and related compounds (i.e., short-chain ethoxylates and carboxylic acid derivatives), are byproducts of the microbial breakdown of APEOs, such as occurs during sewage treatment (Ahel et al., 1994; Giger et al., 1984).

In a study of effluents from sewage treatment works in England, APEOs were identified as a major estrogenic contaminant in the effluent from Marley STW (which receives inputs from wool-scouring

plants) (Waldock et al., 1997) and as a more minor estrogenic contaminant in the Chelmsford effluent (Rodgers-Gray et al., 2001). Along the Aire, wool-scouring plants use APEOs in large amounts to wash grease from the fleeces, and the liquor is then discharged via Marley STW into the river. In 1994, the concentration of NP in the effluent alone exceeded 300 µg/L (EA, 1998). In Switzerland, NP in effluents from sewage treatment works has been measured at concentrations between 36 and 202 µg/L (Stephanou and Giger, 1982), and in Italy concentrations of up to 4 µg/L 4-*tert*-NP have been found (Dicorcia et al., 1994). In river water downstream from the Marley STW on the River Aire, concentrations of NP were recorded up to 180 µg/L, and between 24 and 53 µg/L of dissolved NP was present along significant stretches of the river (EA, 1998). Concentrations of APEOs, however, are now considerably lower in these stretches of the River Aire as a consequence of the implementation of tighter discharge limits and effluent treatment processes at the source (Sheahan et al., 2002a,b). Although concentrations of NP in industrial effluents can exceed 100 µg/L, in most of the English rivers studied concentrations of NP are typically less than 10 µg/L. In the River Lea, for example, measured concentrations of NP were between 0.2 and 9 µg/L. NP in rivers in Switzerland has been reported at concentrations ranging between 0.1 and 0.3 µg/L (Ahel et al., 2000). Concentrations of NP in river water in Europe are generally higher than those found in the United States. This was illustrated in a survey of 30 rivers in the United States which found that 70% of the sites had concentrations of NP of 0.1 µg/L or less (Naylor, 1995). The limited information on concentrations of octylphenol (OP) in the environment indicates that they are generally lower than those for NP; for example, in the River Lea in the United Kingdom, OP was measured at 0.4 µg/L.

It should be emphasized that NP and OP are only two of the degradation products of APEOs and that short-chain NPEOs and NP carboxylates (NPECs) have also been shown to possess some estrogenic activity (Jobling and Sumpter, 1993) and may contribute to the overall estrogenic effect seen in the River Aire and elsewhere. In the Chelmer effluent in the United Kingdom, NP1EO and NP2EO have been measured at concentrations ranging between <0.2 µg/L (below detection limit) and 8.9 µg/L (Rodgers-Gray et al., 2000). Short-chain APEOs are metabolized almost exclusively to carboxylic acid derivatives (APECs); therefore, NPECs may be the most environmentally relevant products of alkylphenol polyethoxylate surfactants in terms of their consequence to aquatic organisms. Despite this fact, there are very few reports of the concentrations of NPECs in the aquatic environment in Europe. In one study of the River Glatt in Switzerland, the most abundant alkylphenolic compounds in the river water were nonylphenoxy carboxylic acids; concentrations of NP1EC and NP2EC ranged between 1 and 45 µg/L and between 2 and 71 µg/L, respectively (Ahel et al., 1994). In a study in the United States, the total concentrations of NPECs (NP1EC to NP4EC) measured in 15 paper mill effluents discharging into the Fox River near Green Bay, Wisconsin, ranged from below detection (<0.4 µg/L) up to 1300 µg/L, but were typically less than 100 µg/L (Field and Reed, 1996). The concentrations of NEPCs measured in six STW effluents discharging into the same river ranged from 140 to 270 µg/L. In all cases, NP2EC was the dominant oligomer, constituting 72% and 54% of the total NPECs in the paper mill and STW effluents, respectively (Field and Reed, 1996). The resulting concentration of total NPECs in the downstream Fox River was 13.5 µg/L, and the total NPECs were composed mainly of NP2EC (87.4%) and NP1EC (12.6%) (Field and Reed, 1996).

Concentrations of alkylphenols tend to be higher in sediments than in the water itself, because they are lipophilic and because biodegradation of these compounds is usually poor under anaerobic conditions (Ball et al., 1989). The concentrations of NP and NP1EO in the sediment may be 2 to 3 orders of magnitude higher than the levels measured in river water (Naylor, 1995). As an example, Naylor reported average concentrations of 162 mg NP per kg and 18 mg NP1EO per kg in sediments, compared with 0.12 mg NP per L and 0.09 mg NP1EO per L in river water (Naylor, 1995). Similarly, in the River Glatt in Switzerland, the ratio of NP concentrations observed in sediment to concentrations in the water ranged between 364:1 and 5100:1, indicating preferential association of NP to sediments (Ahel et al., 1994). Many aquatic organisms, such as polychaete worms, many mollusks, nematode worms, and some fish (e.g., eels) actually live in the sediment, and other species (including many species of fish) live on the surface of sediments. In turn, these animals may receive considerable direct exposure to alkylphenolic chemicals via the sediment as well as through the water.

Estrogenic Activity in Fish

Reports describing the estrogenic effects of alkylphenols in mammals can be found as far back as 1938, when it was reported that 100 mg subcutaneous injections of either 4-propylphenol or 4-*tert*-pentylphenol induced estrus in ovariectomized rats (Dodds and Lawson, 1938), thus mimicking the activity of estradiol-17β. We now know that octylphenol, 4-*tert*-NP, NP2EO, and NP1EC are all estrogenic to fish and have pronounced stimulatory effects on vitellogenin synthesis and can result in modifications in somatic growth rate and gonad growth (Ashfield et al., 1998; Jobling et al., 1996). 4-*tert*-Nonylphenol induced elevations in plasma VTG at a threshold concentration of between 6.4 and 10 μg/L in rainbow trout after 2- to 3-week exposures (Thorpe et al., 2001, 2003; Jobling et al., 1996), 5.4 μg/L in the sheepshead minnow (*Cyprinodon variegatus*) after 42-day exposures (Hemmer et al., 2001), and between 0.65 and 8.1 ± 1 μg/L in male fathead minnows after 3-week exposures (Harries et al., 2000). Exposure to 4-*tert*-NP also causes disruptions in gonadal development that include alterations in the testis structure (Kinnberg et al., 2000) and induction of ovo-testis. Studies on the biological activity of p-NP in fish have shown that exposure of juvenile Japanese medaka (*Oryzias latipes*) to 50 μg p-NP per L in the water can result in the induction of hermaphroditism (ovo-testis), and exposure to a concentration of 100 μg p-NP per L results in complete feminization (Gray and Metcalfe, 1997). Furthermore, in fathead minnows, a 3-week exposure to 4-*tert*-NP at concentrations in the water between 0.65 and 8.1 ± 1 μg/L resulted in a significant decrease in fecundity in females and a reduction in the development of secondary sexual characteristics in males (Figure 25.13) (Giesy et al., 2000; Harries et al., 2000). Similarly, exposure of mosquitofish (*Gambusia holbrooki*) to 50 μg 4-*tert*-NP/L from 3 days post-parturition to sexual maturity resulted in all the population having female secondary sexual characteristics (Dreze et al., 2000). In addition to the effects on testis development, exposure to NP is suspected to impact sperm quality in fish. This was demonstrated in the Japanese medaka, where a short-term exposure to 20 and 100 μg/L NP resulted in a reduced number of motile sperm (Kawana et al., 2003). Some of the effects of 4-*tert*-NP on sexual development have been shown to occur at the level of the pituitary gland, resulting in an altered synthesis/release (inhibition) of follicle-stimulating hormone (Figure 25.13) (Harris et al., 2001). Exposure of zebrafish embryos to 4-*tert*-NP indicated that disruptions in gonad development may be related to an altered distribution of primordial germ cells prior to development of a morphologically distinct gonad (Willey and Krone, 2001). In the sea bass (*Dicentrarchus labrax*), exposure to NP caused alterations in the activity of phase 1 and 2 biotransformation enzymes in the liver, suggesting that the capability of the exposed fish for detoxification of chemicals and also metabolism of endogenous steroid hormones is likely to be compromised (Vaccaro et al., 2005). Similar results were also described by Hughes and Gallagher (2004) following exposure of largemouth bass (*Micropterus salmoides*) to 4-NP. Recent studies in Atlantic salmon (*Salmo salar*) indicated that short-term exposure to 4-NP may impair smoltification and survival and delay subsequent downstream migration (Madsen et al., 2004). The effects observed may be mediated at least in part by induction of changes in the normal behavior of the exposed fish, specifically impacting on the ability to migrate and avoid predators. Further studies investigating the physiological pathways mediating the effects of 4-NP exposure on smoltification in Coho salmon (*Oncorhynchus kisutch*) were not able to detect any changes in thyroid hormones (Keen et al., 2005).

Octylphenol has been shown to induce elevations in plasma VTG in rainbow trout and male roach at threshold concentrations of 3 μg/L (Jobling et al., 1996) and between 10 and 100 μg/L (Routledge et al., 1998), respectively, a potency of between 1/100th and 1/1000th that of estradiol-17β (Routledge et al., 1998). Higher concentrations of OP have been shown to inhibit testis development in the summer flounder (*Paralichthys dentatus*) (Mills et al., 2001) and inhibit spermatogenesis and induce oocytes in the testis of male the Japanese medaka (Gronen et al., 1999). Exposure of medaka to concentrations of 4-*tert*-OP between 25 and 50 μg/L for 6 months from 1 day post-hatch to sexual maturity resulted in reduced reproductive success, which was believed to be a consequence of effects on courtship behavior (Gray et al., 1999). Some of the disruptive effects of 4-*tert*-OP on reproduction in fish may occur as a consequence of altered expression of P450 aromatase (Navas and Segner, 2000).

In summary, the information currently available suggests that the levels of alkylphenolic chemicals in certain rivers, such as the River Aire, are high enough to cause not only VTG induction but also reproductive effects in fish. In general, it would appear that the concentrations of nonylphenol and

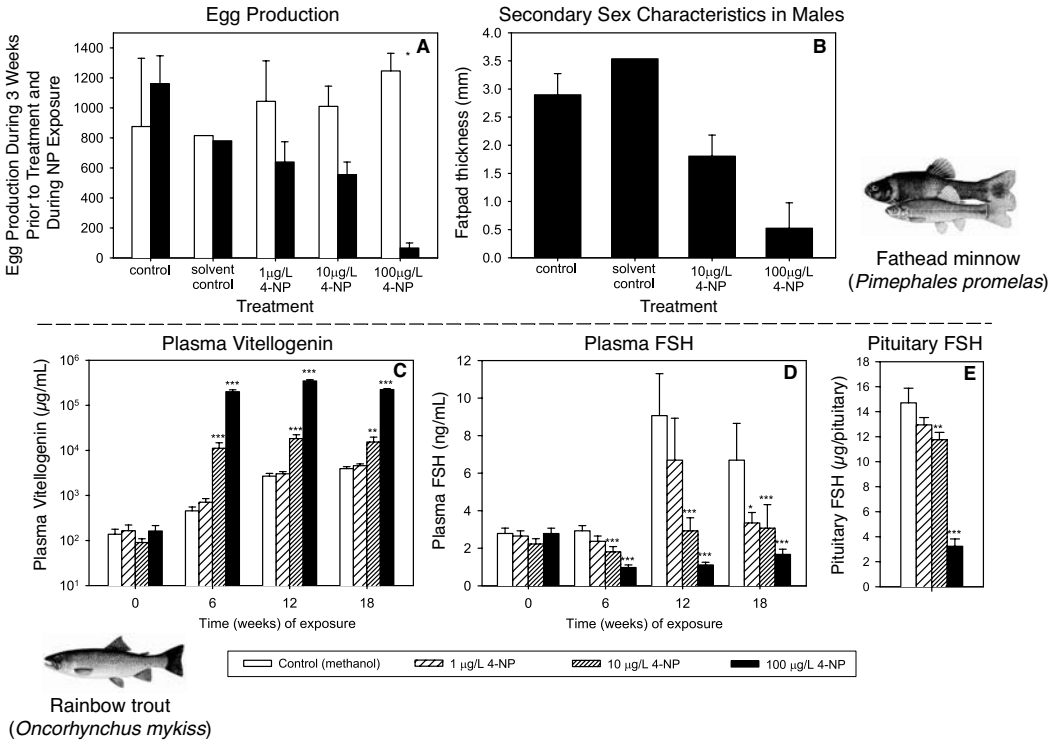

FIGURE 25.13 Effects of 4-*tert*-nonylphenol on reproductive function in the fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*). (A) Egg production in fathead minnows for a 3-week period prior to and 3 weeks during chemical exposure. Asterisk represents significant differences between the before and during exposure groups ($*p < 0.05$). (B) Fatpad thickness in male fathead minnows exposed to 4-*tert*-NP for 3 weeks. (Parts A and B are adapted from Harries, J.E. et al., *Environ. Sci. Technol.*, 34, 3003–3011, 2000.) (C) Plasma vitellogenin concentrations, (D) plasma follicle-stimulating hormone, and (E) pituitary follicle-stimulating hormone in female rainbow trout exposed to 4-*tert*-NP for 18 weeks. Asterisks represent significant differences from the solvent (methanol) controls ($**p < 0.01$, $***p < 0.001$). (Parts C, D, and E are adapted from Harris, C.A. et al., *Environ. Sci. Technol.*, 35, 2909–2916, 2001.)

octylphenol alone in typical English rivers are not high enough to cause estrogenic or reproductive effects in fish; however, the presence of other degradation products of APEOs (such as the relatively water-soluble NPECs) may also contribute to the overall estrogenic activity in the water, but they have received little study. Indeed, in some rivers in the United Kingdom, the combined concentrations of NP, OP, NP1EO, and NP2EO are high enough to induce VTG induction or disruption in gonadal development and reproduction (e.g., River Aire, 15 to 76 μg/L; River Mersey, 6 to 11 μg/L; Tees estuary, up to 72 μg/L) (Blackburn et al., 1999). Furthermore, it should be reiterated that care needs to be taken when the results from short-term (3-week) *in vivo* laboratory exposures (designed to investigate the biological potency of single chemicals) are used to extrapolate their long-term effects on fish in the riverine environment.

The estrogenic potency of alkylphenolic chemicals (and other endocrine-disrupting chemicals) will also depend on whether they are bioconcentrated from the water column or bioaccumulate in the food chain. Alkylphenols are lipophilic chemicals, and fish can absorb them from the water, sediment, and food and retain them in their lipid reserves. Examples of bioconcentration factors (BCFs), which are the ratio of the concentration determined in the organism to the concentration detected in the water column, that have been calculated for NP in fish include 90 to 125 in rainbow trout after a 3-week exposure to 4-*tert*-NP (Blackburn et al., 1999), 280 in salmon (McLeese, 1981), 350 in fathead minnow (Naylor, 1995), 1134 in roach fry exposed for 19 days (Ferreira-Leach and Hill, 2000), and 1300 in sticklebacks (Ekelund et al., 1990). Reported BCFs for OP in a variety of fish species range from 167 to 282 (Tsuda

et al., 2001). There is also evidence for biomagnifications (concentration up the food chain) of alkylphenols in fish (Shiraishi et al., 1989). As fish use lipid rather than carbohydrate as an energy source (Babin and Vernier, 1989), alkylphenols stored in the fat will be mobilized during periods of high energy demand, and periodically this will result in higher exposure concentrations in the fish than in the environment, thus increasing any biological effect of the accumulated compound. Indeed, the bioaccumulative property of 4-*tert*-NP has been shown to result in a 100-fold higher estrogenic potency *in vivo* than occurs *in vitro* (Jobling and Sumpter, 1993; Jobling et al., 1996). An additional consideration for lipophilic chemicals, such as alkylphenols, is that they may concentrate in developing oocytes in fish (because the yolky eggs sequester large amounts of lipid) (Ungerer and Thomas, 1996). Alkylphenols locked up in the egg are likely to be released to the developing embryo during the utilization of these lipid reserves at a very sensitive time in development when the sex is labile (Adkinsregan et al., 1995). In view of this, it is possible that exposure to alkylphenols need only occur during the period of ovarian recrudescence in adults to subsequently have permanent (organizational) developmental effects in the subsequent offspring.

Mixtures Effects

As a final consideration on the chemicals of concern in English rivers, it should be emphasized that the laboratory evidence presented to date for the estrogenic/reproductive effects of steroid estrogens and alkylphenolic chemicals has been based on exposures to single chemicals. In reality, wild fish in English rivers will be exposed to a mixture of estradiol-17β, estrone, and ethinylestradiol. In some instances, this mixture will also include effective doses of alkylphenolic chemicals and possibly other estrogenic compounds, too. Indeed, analysis of bile extracted from fish (both rainbow trout and roach) exposed to U.K. treated sewage effluents has shown that a mixture of estrogenic chemical is taken up and concentrated in exposed fish (Gibson et al., 2005). These chemicals include steroid estrogens, equine estrogens (used in hormone replacement therapy), alkylphenolic chemicals, bisphenols, and plasticizers. *In vivo* studies have shown that estrogenic chemicals in combination can indeed have additive effects in fish. A combination of estradiol-17β and estrone, each at a concentration of 25 μg/L, was shown to produce an induction of VTG greater than a response to 50 μg/L estradiol-17β (Routledge et al., 1998). More recent and comprehensive studies using VTG induction in juvenile female rainbow trout, analyzed using the model of *concentration addition*, have shown that binary mixtures of natural and synthetic steroid estrogens and 4-*tert*-nonylphenol all have additive effects (Thorpe et al., 2001, 2003). Adding a further dimension, and complication, to this work, recently we have also found that many U.K. STW effluents are also strongly antiandrogenic (the causative chemicals have yet to be identified), and this activity will further increase the feminizing effects of these estrogenic effluents. These results illustrate the need to consider the concentrations of all the different estrogens (and antiandrogens) in the effluent, rather than its individual components alone, if a realistic estimate of the biological potency and potential estrogenic impact of effluents in wildlife is to be determined.

The Dimension of the Estrogenic Problem in U.K. Rivers

England and Wales have more than 70,000 consented discharges into freshwaters. These range in size from flows of over 150,000 m^3/day to flows of less than 5 m^3/day, and most of these discharges (82%) are from sewage treatment works. Nationally, over 10,000 megaliters are collected by water companies every day for STW treatment in the United Kingdom, and this figure is increasing by about 2% per year, consistent with the increase in water supplied. Most of the influent to sewage treatment works is domestic in origin. A national figure for the content of trade effluent in sewage treatment works in the United Kingdom is 10% of total discharges. Altogether, around 6500 sewage treatment facilities are in the United Kingdom, and treatment of sewage varies depending on the history of management, needs of the receiving waters, and past legislation. In theory, discharges are consented with quality standards attached to minimize the effects of pollution on receiving waters. In practice, however, many consents are historic,

and a lack of investment in sewage treatment works before privatization in 1989 has led to widespread failure to meet consent conditions. To illustrate this, in the mid-1960s, 60% of sewage works failed to meet their consents, but by 1990 only 10% were failing. In 1995, less than 4% of sewage treatment works in the United Kingdom failed to meet their consent conditions.

Discharges from sewage treatment works into rivers are based primarily on the organic load of the effluent. Historically, STW discharge consents in the United Kingdom were based on a 1:8 dilution by the river at the point of discharge, a ratio that was considered sufficient to reduce the amount of biological oxygen demand (BOD), chemical oxygen demand, and suspended solids to acceptable levels (OECD, 1994). Effluent dilution studies in England (using caged trout) found that dilutions between an excess of 1:2 (Harpenden STW, River Lea) and 1:4 (Chelmsford STW, River Chelmer) were required to reduce estrogenic activity to a no-effect threshold (Harries et al., 1999). Based on using the short-term (3-week) caged-trout bioassay, therefore, the 1:8 discharge consensus would appear to be sufficient to remove estrogen activity in effluents such as the Chelmer and Harpenden. As detailed earlier, however, more recently it has been established that longer term exposure to STW effluents reduces the effective threshold concentration to induce an estrogenic (vitellogenic) response in fish, and exposure of roach to the Chelmer effluent for 4 months resulted in an induction of VTG in male fish at an effluent concentration of only 9.4 ± 0.9% (Rodgers-Gray et al., 2000). The 1:8 dilution discharge consensus, therefore, is unlikely to be sufficient dilution of the effluent for wild fish living in the River Chelmer to avoid exposure to biologically active estrogen, and this is probably true for many, if not most, other rivers receiving STW effluent discharges in the United Kingdom. Furthermore, some of the consents assigned to British rivers are now out of date, because in certain rivers (such as the River Lea) up to 80% of the river volume is made up of STW effluent discharge at times of low flow (usually during the summer months; see Figure 25.14) (NRA, 1992), and this figure can be even higher in periods of drought, as experienced in the United Kingdom during 1995 and 1996. Indeed, nationally in the United Kingdom, the effluents from many large sewage treatment works (with a population equivalent of >10,000) have a dilution ratio of less than 1 in 10 before they are discharged into rivers (see Figure 25.14). Furthermore, the summer months (when the river flows are at their lowest and the effluent concentrations are at their highest) coincide with the time when many juvenile cyprinid fish (such as the roach) are undergoing sexual differentiation, a time when they are likely to be most vulnerable to endocrine disrupting chemicals. The conditions seen for effluents discharged into rivers in England are far from unusual in Europe. Indeed, despite the fact that many rivers in England contain high concentrations of treated effluent, their chemical and biological quality are considerably higher than the majority of other European countries, with less than 25% having a BOD exceeding 3.5 mg O_2/L (OECD, 1994). In the United Kingdom, around 95% of the organic polluting load is removed from the influent by the sewage-treatment process before the effluent is discharged. This level of treatment is among the highest in OECD countries (probably reflecting the density of population) and is exceeded only by Germany (OECD, 1994). It is likely, therefore, that the problems associated with estrogens discharged in STW effluent into rivers in England occur widely in other European countries.

Conclusions

In summary, natural and synthetic steroidal estrogens, and in some instances alkylphenolic chemicals, have been identified and confirmed as being key causative agents for the vitellogenic responses and disruptions in duct development seen in fish in English rivers. It is likely that these chemicals are also responsible for (or at least contribute to) the evolution of oocytes in the testis of males (feminization of male germ cells) in wild fish in England, given that: (1) high doses of estrogen can induce oocytes in male fish, and for ethinylestradiol the effective concentrations for doing so are similar to those found in some of the more potent effluent discharges; (2) exposure to exogenous steroid estrogens and some alkylphenols alters the normal balance of endogenous hormones (e.g., steroids and gonadotrophins) and enzymes (e.g., P450 aromatase) that mediates sexual differentiation and germ-cell development; (3) estrogenic effluents from sewage treatment works can make up a considerable proportion of the river flow in English rivers; (4) fish living in many rivers are likely to be exposed to these estrogenic compounds

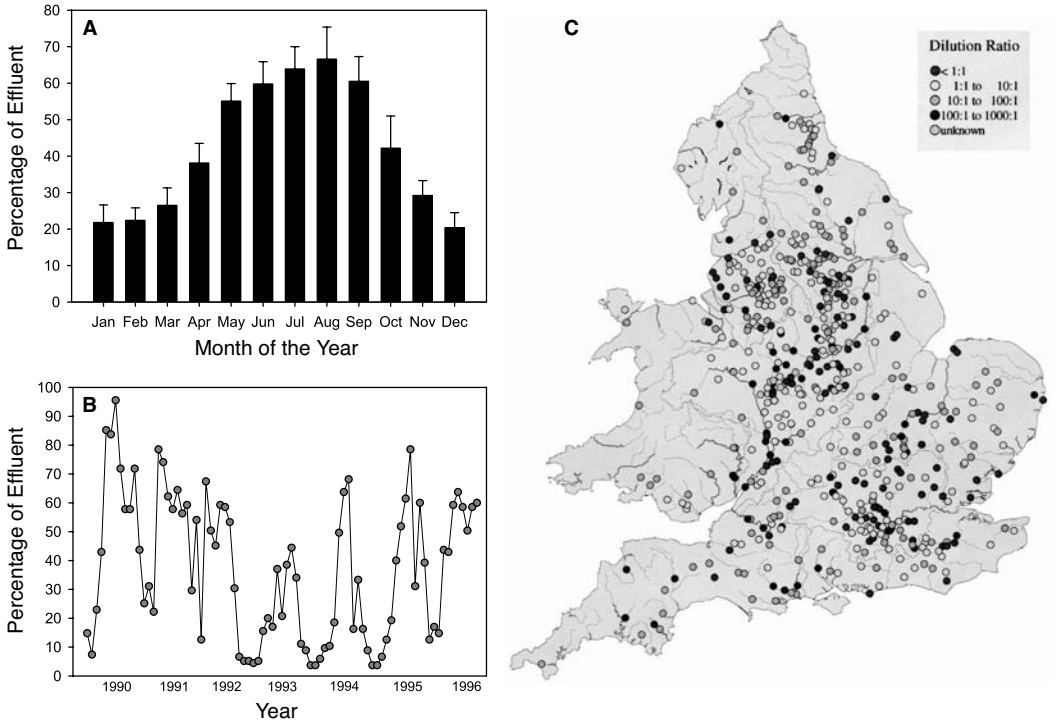

FIGURE 25.14 Representation of the percentage flow in the River Lea (U.K.) comprised of treated sewage effluent in (A) a single year and (B) over 6 years; (C) dilution ratio of effluents from large sewage treatment works (with population equivalent of greater than 10,000) discharging into U.K. rivers.

for considerable periods of time or possibly all of their lives; (5) wild fish in English rivers are exposed to a mixture of estradiol-17β, estrone, ethinylestradiol, alkylphenolic chemicals, and, indeed, any other estrogenic chemicals present, some of which have been shown to be additive in their estrogenic effects; and (6) both alkylphenols and steroid estrogens bioaccumulate in fish which increases their biological effects. The approach adopted to identify the causal agents of estrogenic effects in fish in English rivers, combining biological studies (in both the field and laboratory) with high-quality analytical chemistry, proved to be most successful. These studies on English STW effluents, however, were made easier by knowing that the major compounds of concern were estrogenic in nature.

To alleviate the biological problems associated with steroid estrogens in English rivers, higher levels of dilution of STW effluent are required before it is discharged. This is not easily overcome, however, given the drought problems that have occurred in recent years and the ever-increasing demand for clean water, which together have resulted in low flows in many rivers in England. Approaches to tackling the steroid problem in sewage treatment works in the United Kingdom, therefore, are either an improvement in the treatment processes *per se* or the implementation of systems that specifically remove or degrade steroids in the treatment works. The problems associated with the discharge of estrogenic alkylphenolic compounds are more easily addressed than those for steroidal estrogens. Where high quantities of alkylphenolic compounds are in use, a program of tighter regulation for their discharge or a switch to alternative (non-estrogenic) surfactants is needed. Indeed, the potential success of such a program is illustrated in the River Aire, where a reduction in the concentration of estrogenic alkylphenolic chemicals in the river has resulted from changes in textile and wool-scouring practices and a reduced discharge of APEOs. In 1995, concentrations of alkylphenolic chemicals in the effluent from the Marley STW were reduced to approximately 80% compared with 1994 and were reduced a further 15% by 1996; these reductions have resulted in the concomitant decrease in estrogenic responses in caged fish placed in the river downstream of this discharge (Sheahan et al., 1998, 2002a,b; Waldock et al., 1997). To finish on a further positive

note, a so-called "demonstration program," led by the U.K. Environment Agency and involving the water companies, is investigating technologies for the removal of endocrine-disrupting chemicals from treated sewage effluents. It is envisaged that this program will result in the development and implementation of cost-effective improvements in the treatment of STW effluents on a national scale and, in turn, lead to lower discharges of EDCs and a reduction of their impact on wild fish living in U.K. rivers.

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Index

B

C

D

E

F

G

H

I

J

K

L

M

O

P

Q

R

S

T

U

V

W

X

Y

Z

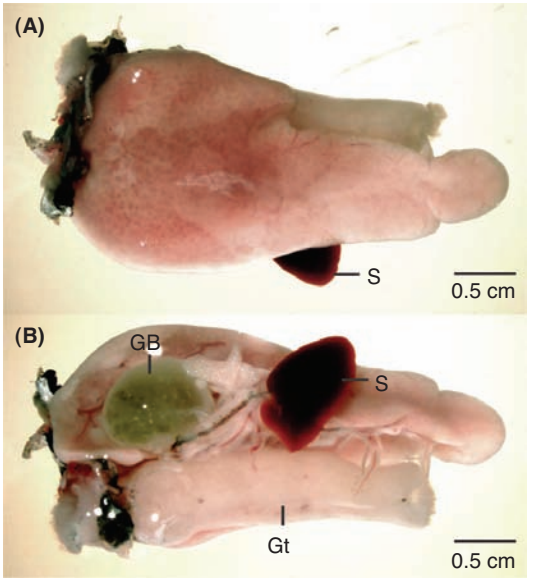

FIGURE 7.1 (A) Ventral view of freshly dissected liver of *Fundulus heteroclitus*. This liver is typical of the compact, single-lobed livers of many teleosts. (B) Dorsal view of the liver shown in A. Between the gall bladder and larger spleen, the hilus of the liver is seen. It is here that the hepatic ducts leave the liver and the arterial and afferent venous blood supplies enter the organ. GB, gall bladder; Gt, gut; S, spleen.

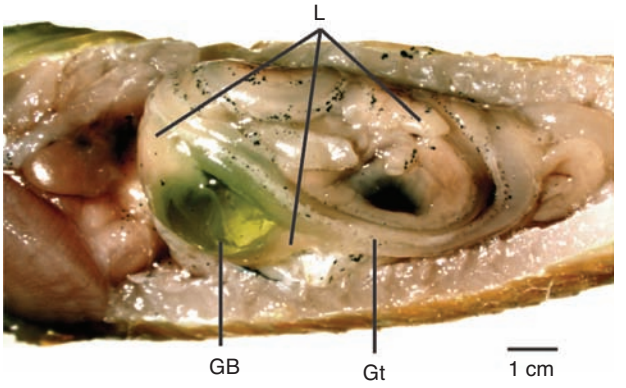

FIGURE 7.2 Freshly dissected visceral mass from goldfish (*Cyprinus carpio*) illustrating the liver pattern of some teleosts in which the organ is comprised of multiple lobes or extensions between coils of the intestine. The black material is due to melanocytes in the visceral peritoneum of this abdominal cavity. Establishing a precise liver weight or dissecting the organ for biochemical or molecular analyses would prove difficult with livers of this form. Arrows point to portions of the liver in close proximity to the elongated intestinal tract. GB, gall bladder; Gt, gut; L, liver.

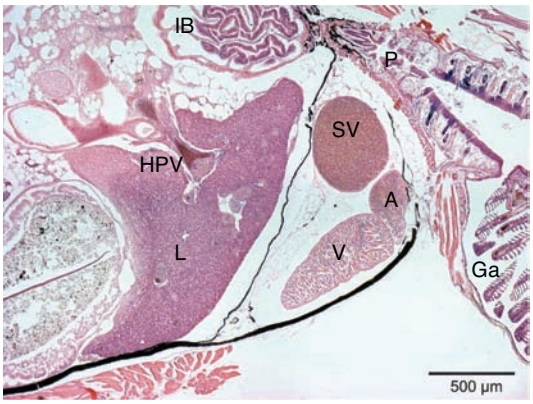

FIGURE 7.3 Hematoxylin and eosin stain of section through adult medaka (*Oryzias latipes*). Rostral structures are oriented toward the right side of the field; dorsal is top and ventral bottom. Parasaggital section shows portions of the abdominal cavity, pericardial cavity, branchial chamber, and pharynx. A, entry to aorta; Ga, gill arch with primary lamellae attached; HPV, hepatic portal vein in liver hilus; IB, intestinal bulb; L, liver; P, pharyngeal mucosa with teeth; SV, sinus venosus; V, ventricle. See micron bar for magnification.

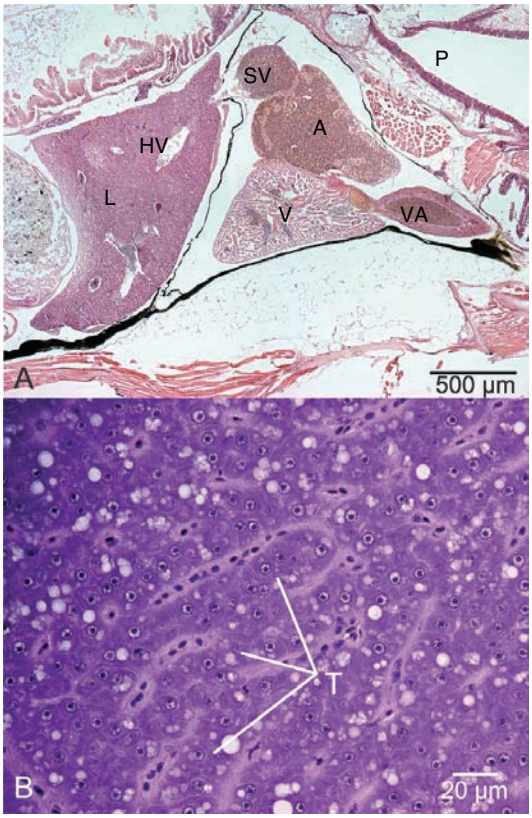

FIGURE 7.4 (A) Stain and orientation are identical to Figure 7.3. A, atrium; HV, hepatic vein conducting blood from liver to sinus venosus (SV); L, liver; P, pharynx; V, ventricle; VA, ventral aorta. Melanin is in parietal pericardium and parietal peritoneum. See micron bar for magnification. (B). Section of adult female liver shows basophilic cytoplasm overlying endoplasmic reticulum (purple areas of hepatocytes). Large vacuoles are fat. This appearance is that of the reproductively active female with vitellogenin production. T, hepatic tubule formations.

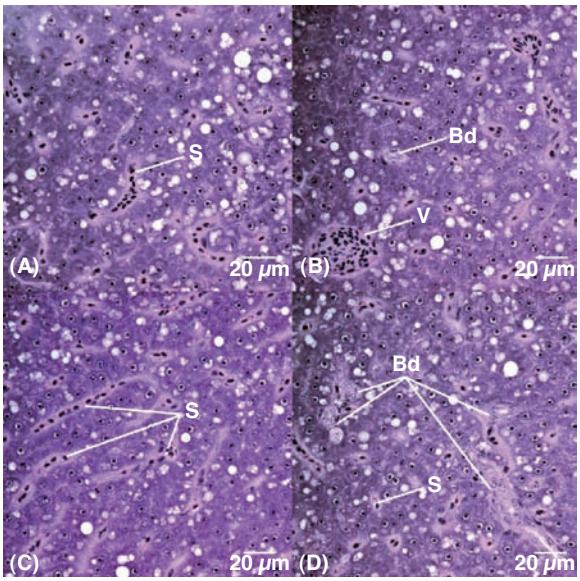

FIGURE 7.5 High-resolution light micrographs of medaka liver showing elements of hepatic architecture. (A) Hepatic tubules comprised of hepatocytes and biliary epithelial cells are partially separated by sinusoids (S). No lobular architecture is apparent. Rounded white structures are lipid vacuoles. Lipid was removed during alcohol dehydration in processing. (B) Hepatic sinusoids contain nucleated red blood cells (top right of field). Larger venule (V) has no arterial or biliary structure associated. (C) This field shows sinusoids (S) between which are found hepatic tubules in longitudinal array. Note the double row of hepatocytes making a single tubule. Tubules are incompletely separated by sinusoids. (D) Region near the hilus of the liver is shown. Intrahepatic bile ducts of varying sizes (Bd) are shown. Note the difference in staining and in nuclear profiles in biliary epithelial cells vesus hepatocytes.

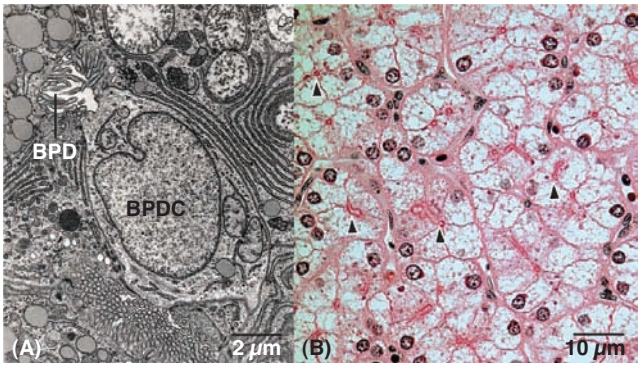

FIGURE 7.8 Canaliculi and transitional zones of intrahepatic biliary system are illustrated. (A) Transmission electron micrograph of liver shows portions of two hepatocytes and one transitional biliary epithelial cell, termed bile preductular epithelial cell (BPDC). This cell shares junctional complexes with hepatocytes and forms portion of wall of bile passageway now termed bile preductule (BPD; a transitional zone). Note the absence of microvilli supplied to bile passageway by the BPDC. (B) Light micrograph of paraffin-embedded sturgeon liver. Hepatocytes of this species contain little stainable material in cytoplasm with H&E stain. Note the eosinophilic margins of the cells at bile canaliculi and their extensions toward tubule lumen (black arrowheads). We seldom see bile canaliculi staining this well in other species.

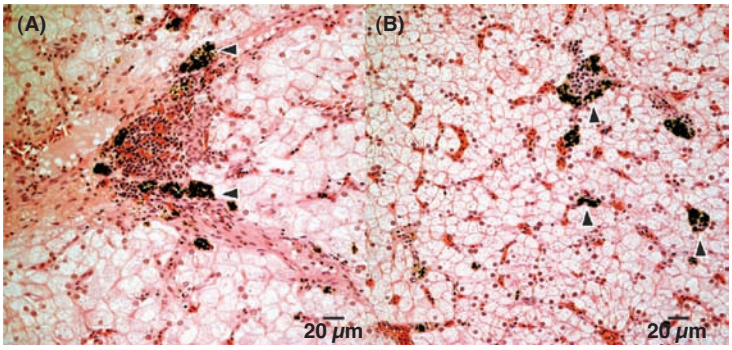

FIGURE 7.11 Hematoxylin and eosin stained sections from liver of juvenile sturgeon. (A) Melano–macrophage aggregates (black arrowheads) are seen in the connective tissue tract at margins of inflammatory focus (eosinophilic granular leukocytes and mononuclear cells). (B) Liver parenchyma has smaller melano–macrophage aggregates in this juvenile. Black arrowheads point to perivascular aggregates.

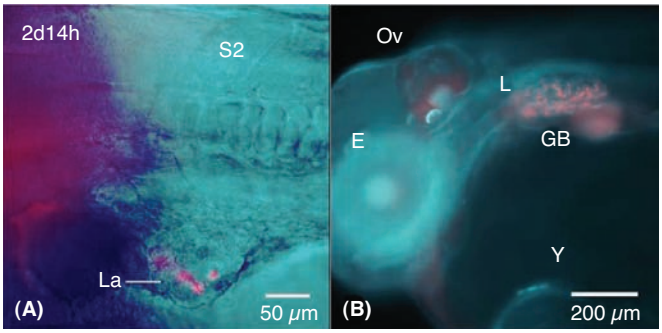

FIGURE 7.12 (A) *In ovo* imaging of medaka liver anlage (La) emerging from the ventral endoderm 62 hours after fertilization. In medaka, this occurs below the first to third somite (S2). Distinct at this stage of development are hepatic tubule formations, elucidated here with the cytochrome P450-3A substrate 7-benzyloxyresorufin (7-BR). Embryonic medaka exposed to aqueous concentrations of 7-BR exhibit CYP3A activity, indicated by the red fluorescence of resorufin (the metabolic byproduct of CYP3A, via dealkylation of 7-BR) in the tubule lumens of the developing liver (red punctuate features). (B) By 5 days after fertilization, the liver (L) of medaka is found in a left lateral orientation, with the gall bladder (GB) at the liver caudal margin. Red fluorescence in the liver and gall bladder is the fluorescent CYP3A byproduct resorufin. Here, the fluorophore is seen in transit through the intrahepatic biliary passageways of the liver, with concentration in the gall bladder. E, eye; Ov, otic vesicle; Y, yolk (sac).

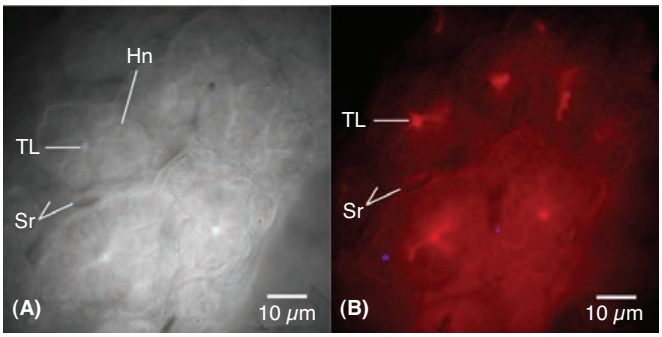

FIGURE 7.13 *In vivo* microscopy of dechorionated medaka embryos 5 days after fertilization; tubular architecture in developing liver of medaka. (A) Autofluorescence of hepatic parenchyma (widefield fluorescence microscopy). Six to eight hepatocytes can be seen to, in transverse section, form the hepatic tubule. The apical membranes of hepatocytes form the tubule lumen, a central biliary passageway. Hn, hepatocyte nucleus; Sr, sinusoid with red blood cells; TL, tubule lumen. (B) Same image as A but showing TRITC fluorescence of 7-benzyloxyresofufin (7-BR) (red). 7-BR-exposed medaka embryo shows fluorescence of resorufin in lumens of hepatic tubules and in canaliculi, thus providing *in vivo* evidence for CYP3A metabolic activity and concentrative transport of fluorophore from the sinusoid to the tubule lumen.

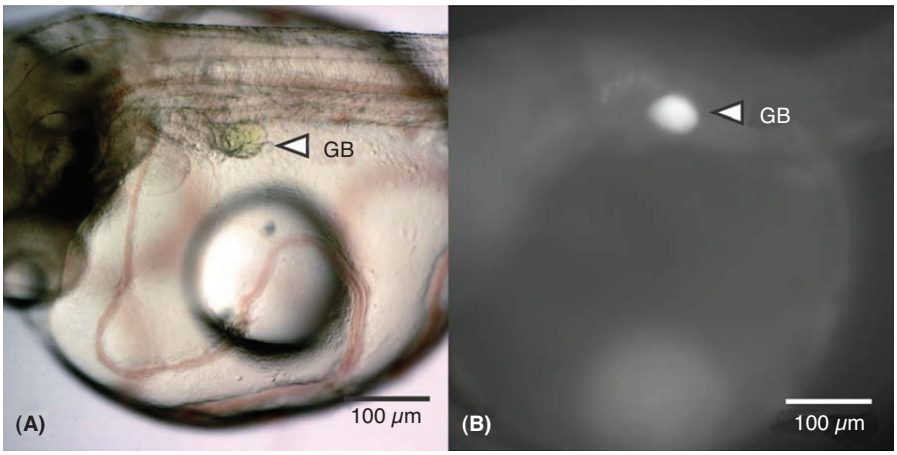

FIGURE 7.14 *In vivo* microscopy (brightfield illumination) of dechorionated medaka embryo. (A) Yellow/green pigment in the gall bladder (GB) is seen. This signifies bile synthesis and export. The liver is in left upper abdominal cavity immediately dorsal to lipid droplet. The circuitous left duct of Cuvier is shown, and a portion of the media yolk vein is at the caudal margin of the yolk sac. (B) Same orientation as in A, demonstrating autofluorescence in the gall bladder and to a lesser extent in the intrahepatic passageways.

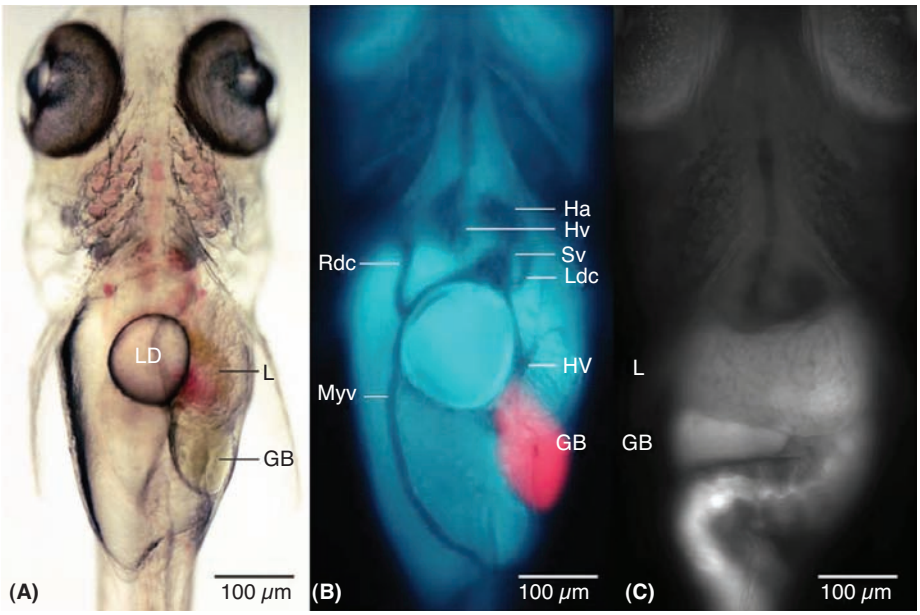

FIGURE 7.15 A and B are ventral view of medaka at 10 days after fertilization. (A) Note right half of abdominal cavity is largely filled by yolk sac showing evidence of utilization of yolk. (B) DAPI/TRITC composite showing vasculature at this developmental stage. Note that the large rounded lipid droplet is maintained despite loss of most of the yolk sac. (C) Medaka at 20 days after fertilization revealing adult phenotype. Note depletion of the lipid droplet (absence). The liver is in the ventral rostral portion of abdominal cavity and partially covers the gall bladder. Autofluorescence in the gut is largely due to algae. The liver shows an extensive vascular network (dark nonfluorescent sinuous lines). GB, gall bladder; Ha, atrium of heart; HV, hepatic vein; Hv, ventricle of heart; L, liver; LD, lipid droplet; Ldc, left duct of Cuvier; Myv, median yolk vein; Rdc, right duct of Cuvier; Sv, sinus venosus.

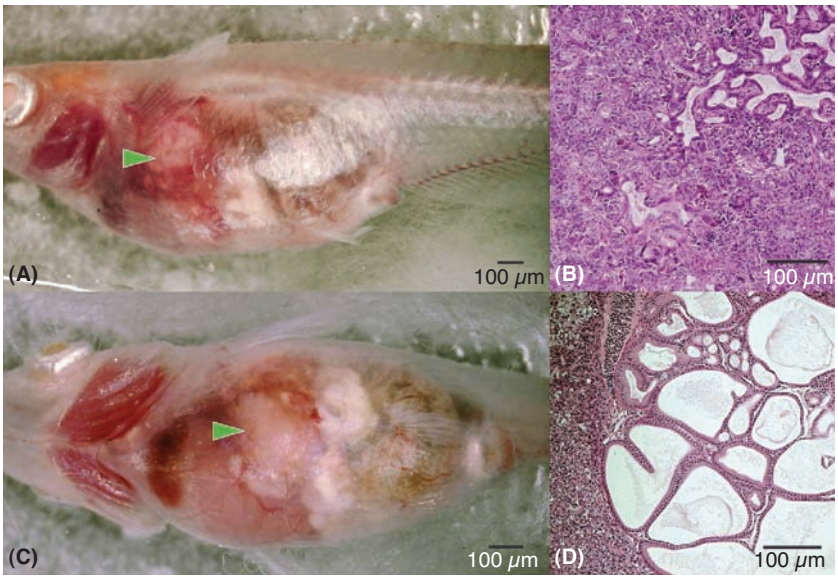

FIGURE 7.16 *In vivo* imaging of tumor formation in STII medaka (brightfield microscopy). Neoplastic response following early life stage exposure of STII medaka to the reference hepatocarcinogen diethylnitrosamine (DEN). Aqueous bath exposure was of 24-hour duration at 100 ppm DEN. Fish were exposed as 14-day-old hatchlings and followed serially in a noninvasive manner for 10 months. At 10 months after exposure, a subset of cohorts developed hepatic tumors (green arrowhead). (A, C) *In vivo* imaging (brightfield) of hepatic tumor formation (green arrowheads) in DEN-exposed medaka; shown is an enlargement of the total liver mass, approximately 18% of the total body length. After anesthesia, the liver was removed and processed for histopathology. Hepatic neoplasm showed mixed hepatocellular (B) and cholangiocellular (D) carcinomas. Focus of biliary hyperplasia was seen in the same liver; here, a single layer of biliary epithelium lines large cystic spaces in the liver (D). The opaque white tissue in the brightfield images (A and B) is ovary; the gut occupies caudal most region of the abdominal cavity.

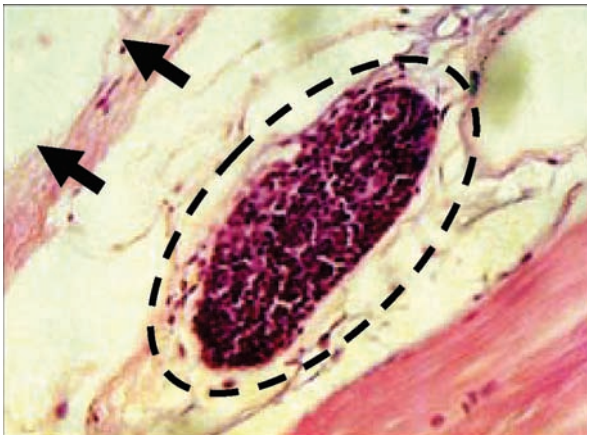

FIGURE 11.1 Teleost thymus as represented by the Japanese medaka (*Oryzias latipes*). Note the close proximity of the organ (dashed circle) to the opercular epithelium (arrows). Hematoxylin and eosin-stained light micrograph (100× magnification).

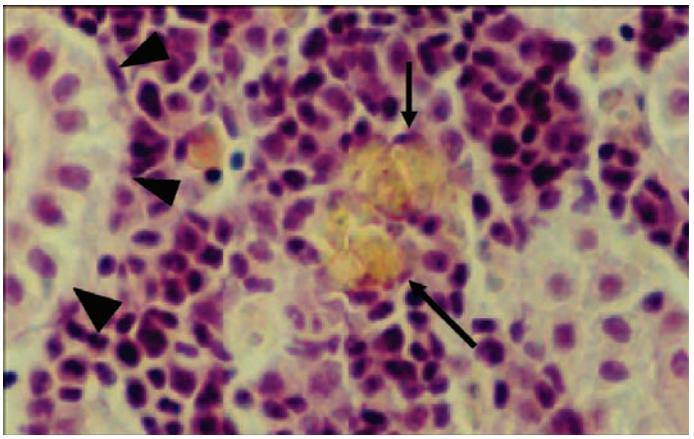

FIGURE 11.2 Teleost anterior kidney as represented by the Japanese medaka (*Oryzias latipes*). Hematoxylin and eosin-stained light micrograph (100× magnification) demonstrating kidney hematopoietic tissue interspersed between renal tubules (arrowhead). Note the presence of small macrophage aggregates (arrow).

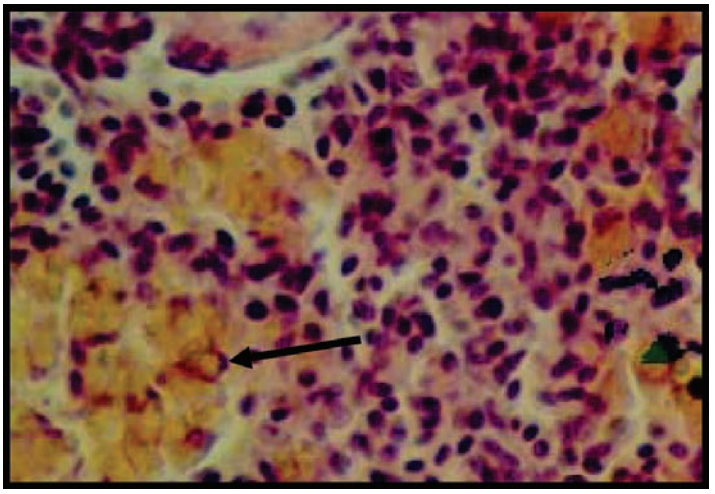

FIGURE 11.3 Teleost spleen as represented by the Japanese medaka (*Oryzias latipes*). Hematoxylin and eosin-stained light micrograph (100× magnification) demonstrating hematopoietic splenocytes. Note the macrophage aggregates (arrow).

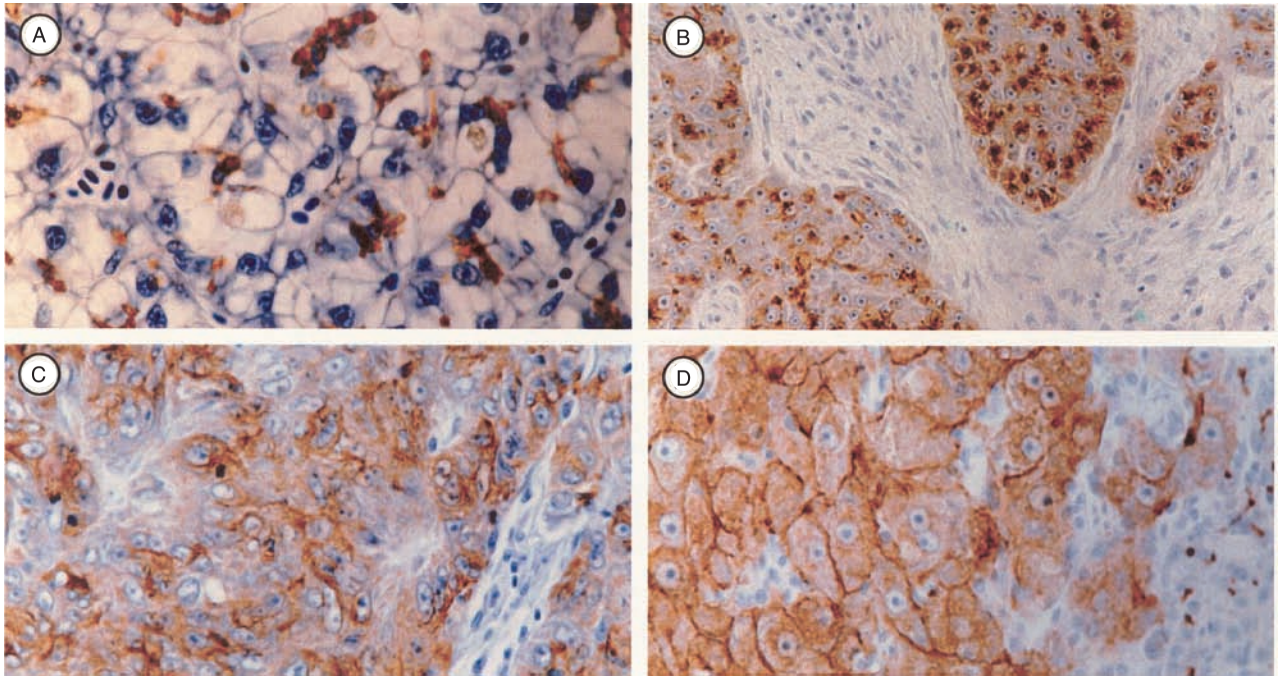

FIGURE 13.15 Immunohistochemical staining of mummichog liver demonstrating expression of P-glycoprotein on (A) canalicular surface of normal liver and (B), (C), (D) overexpression in neoplastic cells of tumor-bearing Atlantic Wood mummichog. (Adapted from Cooper, P.S. et al., *Biomarkers*, 4, 48–58, 1999.)

THE TOXICOLOGY OF FISHES

When looking for a book on fish toxicology, you might find one that discusses the biochemical and molecular aspects, or one that focuses on aquatic toxicology in general. You can find resources that cover human and animal toxicology or ecotoxicology in general, but no up-to-date, comprehensive monograph devoted to the effects of chemical pollution on these organisms has been widely available, until now. Filling this void, ***The Toxicology of Fishes,*** written by recognized experts, is the single most complete coverage of exposure, uptake, and distribution of chemical pollutants by fish and their responses within individuals, species, and communities.

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