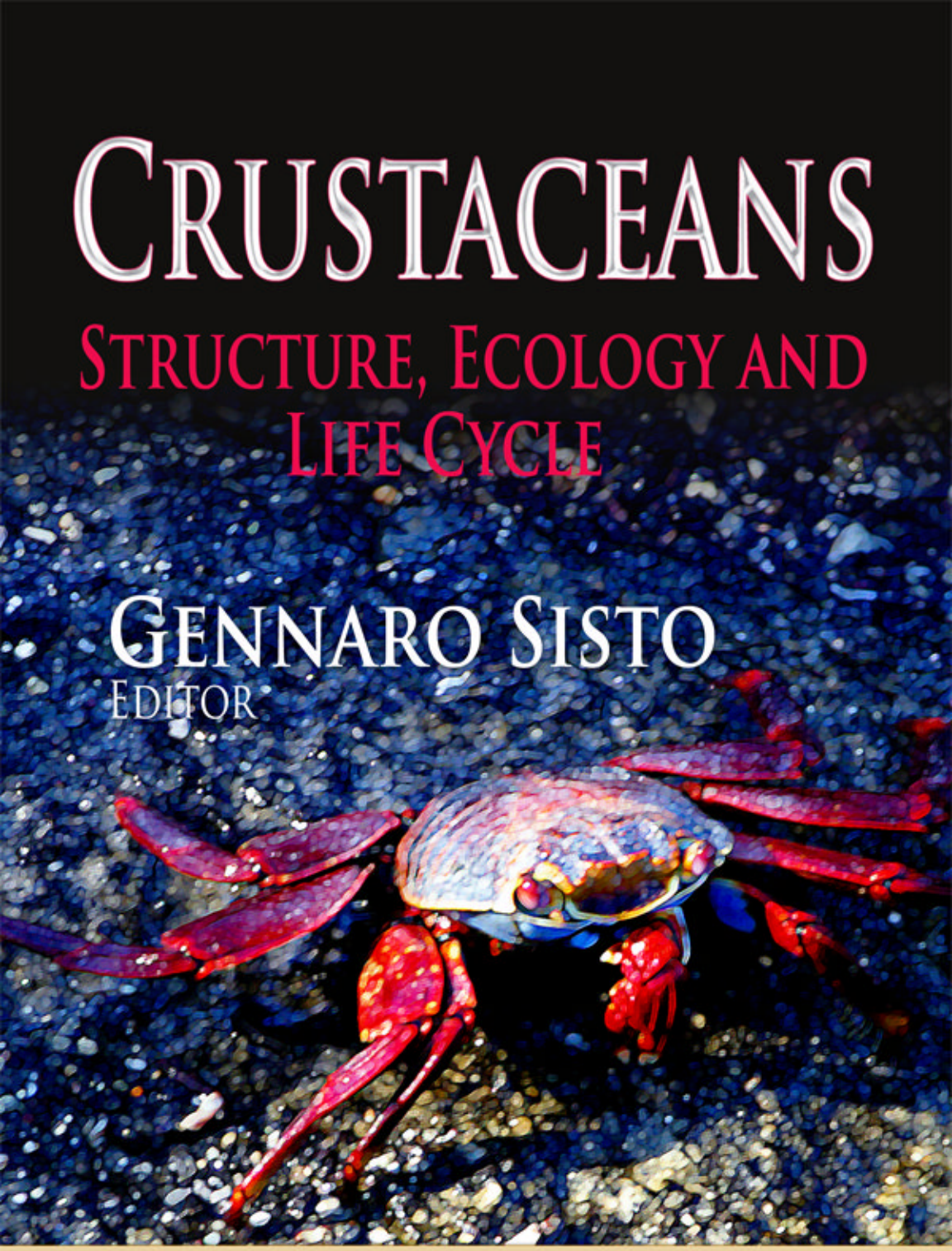
CRUSTACEANS

STRUCTURE, ECOLOGY AND LIFE CYCLE

GENNARO SISTO
EDITOR



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CRUSTACEANS

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PREFACE

In this book, the authors discuss the structure, ecology and life cycle of crustaceans. Topics include the structure, development and evolution of crustacean eyes; the effect of gamma radiation on the extraction of chitosan from the exoskeleton of crustaceans; lipid transport and metabolism in crustaceans; and soft-bottom crustacean assemblages influenced by anthropogenic activities in the Arabian Gulf.

Chapter 1 - Crustaceans exhibit by far the greatest diversity of optical designs of all animal groups. In fact a single crustacean species can exhibit multiple eye types. Classical morphological studies have carefully dissected the morphology and optics of many of these eyes. However, this field has suffered from a lack of genetic data and robust phylogenetic hypotheses. With the advent of strong molecular phylogenies, inexpensive transcriptome sequencing, and developmental genetic techniques that work across species, crustacean eyes stand poised to be a rapidly growing subject of intense study. In this chapter the authors examine the basic structure, development, genetics and evolution of these eyes.

Chapter 2 - Chitin is a structural polysaccharide and is the second most important natural polymer in the world. The main sources exploited are two marine crustaceans, shrimp and crabs. Chitin is a homopolymer of 1-4 linked 2-acetamido-2-deoxy-β-D-glucopyranose, although some of the glucopyranose residues are deacetylated and occur as 2-amino-2-deoxy- β -D-glucopyranose. Despite its huge availability, the utilization of chitin has been restricted by its intractability and insolubility. The fact that chitin is as an effective material for sutures essentially because of its biocompatibility, biodegradability and non-toxicity together with its antimicrobial activity and lowimmunogenicity, points to immense potential for future development. When chitin is deacetylated to

more than 50% of the free amine form, it is referred to as chitosan. Chitosan has attracted considerable interest due to its biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries. In the recent years considerable research efforts have been directed towards establishing a suitable method of extraction of chitosan and approaches to enhance its different properties. Many researchers have focused on application of gamma radiation on chitin and chitosan to obtain a potential bioactive material. This review wishes to provide an overview of the effect of gamma radiation on the extraction of chitosan from exoskeleton of crustacean (prawn shell) based on the authors' and others' latest research results. In addition this review takes a closer look on comparison of physicochemical, thermal and morphological properties of gamma irradiated chitosan with those of the nonirradiated chitosan and potential applications of irradiated chitosan in various fields. From literature survey, it is realized that research activities on uses of gamma radiation on chitosan to improve its biomedical and agricultural applications have increased at the rapid rate. Hence, the present review is timely.

Chapter 3 - In aquatic invertebrates, lipids represent an important source of stored energy, as well as structural components of cellular membranes and other lipoprotein complexes. In the freshwater shrimp *Macrobrachium borellii*, the authors demonstrated that the hepatopancreas, also known as midgut gland, has a high capacity for triacylglycerol biosynthesis, storage and breakdown. When radioactive palmitic acid was injected *in vivo*, most of the label was transported to the hepatopancreas, where triacylglycerol were actively synthesized. The enzymatic activity that initiates glycerolipid synthesis, glycerol-3-phosphate acyltransferase, is located in the mitochondria. In contrast, triacylglycerol synthesis in hepatopancreas microsomal fraction follows the monoacylglycerol pathway. Even though triacylglycerol count for the major lipid class (up to 80% of the total lipids), phosphatidylcholine is exported from the hepatopancreas to other tissues and transported in hemolymph as a high-density lipoprotein (HDL), suggesting that triacylglycerol are stored in the hepatopancreas for energy supply or lipid remodeling. *M. borellii*'s HDL lipid moiety is composed mainly of phosphatidylcholine with minor quantities of cholesterol and triacylglycerol. Lipid transference between hepatopancreas and HDL was studied *in vitro*, confirming that HDL releases free fatty acids to the hepatopancreas, whereas phosphatidylcholine and other phospholipids are liberated from the hepatopancreas to HDL.

Proteins, lipids and carbohydrates present in the vitellus of eggs are important energy and building block sources for the embryo development in ovipara. In aquatic invertebrates, these compounds are usually associated forming lipoproteins called lipovitellins (LV) that function as nutrient sources for the development of the embryo and also satisfy the metabolic larvae needs from their birth to the moment they start feeding on external sources.

In *M. borellii* and other crustaceans, LVs are high-density lipoproteins with phosphatidylcholine as the major lipid and minor quantities of other lipids like triacylglycerol and phosphatidylethanolamine. LVs are originated from a plasma lipoprotein restricted to ovogenic females: vtellogenin (VG). M. *borellii*'s VG lipids are similar quantities of phosphatidylcholine, sphingomyelin, triacylglycerol and phosphatidylethanolamine. During vitellogenesis, VG is endocyted into the ovary, where it is processed and changed into LV. Compared to VG, LV has twice as much phosphatidylcholine as the other lipid classes and less sphingomyelin, suggesting that the lipoprotein processing in the ovary provides LV with different lipid domains specific for its biological function. Due to the fact that some of LV apolipoproteins have been detected inside the developing embryo, it was concluded that LV was consumed. LV transfers to the embryo mainly the lipids loaded inside the ovary (mostly phosphatidylcholine), together with proteins to feed the embryos.

Further studies are needed to determine the lipid transference mechanisms involving the lipoproteins with lipid donor and receptor organs. However, the authors' knowledge about lipid transport and metabolism in crustaceans has evolved in the last few years regarding both plasma and yolk lipoproteins; it sets ground for understanding the lipoprotein function in reproduction and embryo development.

Chapter 4 - The Arabian Gulf is a subtropical semi-enclosed sea characterized by marked fluctuations in sea temperatures and high salinities. Soft substrate benthos forms the largest and most diverse marine ecosystem.

Crustaceans constitute diverse and abundant taxonomic groups of soft-bottom macrofauna. These assemblages provide a useful tool for detecting environmental pollution and disturbance.

This study characterized soft-bottom crustacean assemblages influenced by anthropogenic activities in Bahrain in response to sewage-derived nutrients as well as industrial-derived hydrocarbons and heavy metals. Samples were collected subtidally from a small bay that receives sewage discharge from the largest plant in Bahrain, and off the eastern coastline that is influenced by effluents from the oil refinery and other major industrial factories.

Environmental parameters were synoptically measured, and nutrients as well as heavy metals were analyzed. Although crustaceans were severally impacted by localized sewage pollution, industrial effluents associated with hydrocarbons and heavy metals had more pronounced overall effects on crustacean assemblages represented by lower levels of diversity and abundance.

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Chapter 1

STRUCTURE, DEVELOPMENT AND EVOLUTION OF CRUSTACEAN EYES

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ABSTRACT

Crustaceans exhibit by far the greatest diversity of optical designs of all animal groups. In fact a single crustacean species can exhibit multiple eye types. Classical morphological studies have carefully dissected the morphology and optics of many of these eyes.

However, this field has suffered from a lack of genetic data and robust phylogenetic hypotheses. With the advent of strong molecular phylogenies, inexpensive transcriptome sequencing, and developmental genetic techniques that work across species, crustacean eyes stand poised to be a rapidly growing subject of intense study. In this chapter we examine the basic structure, development, genetics and evolution of these eyes.

I. INTRODUCTION

Crustaceans, like many arthropods, typically have two lateral compound (faceted) eyes and one to several single-lens median eyes. While the

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compound eyes are multi-lensed, they are not merely a collection of median-type eyes but rather a phylogenetically and developmentally distinct type of eye. Compound eyes are image-forming and are the more well-studied of the two eyes as they are large, diverse, and display beautiful and intriguing morphologies. Compound eyes have two general types: the apposition eye, common among Crustacea, and the superposition eye--less common, but more sophisticated.

Superposition eyes exhibit one of three types of optics for resolving a single upright image on a retina: the common refracting superposition eye, the reflecting superposition eye, and the parabolic superposition eye. Median eyes are typically smaller and less structurally charismatic. They are located on the dorsal side of the head and have convex surfaces.

Even among these simple eyes there is significant diversity in the Crustacea. All following eye types are after Land (1981). The simplest type of these is the lenseless pit eyes present as the median eyes in some crustaceans. These pit eyes are little more than small bundles of photoreceptors protected by pigment to give their signal directionality (Elofsson, 2006).

Some median eyes are aquatic lens eyes, such as the unpaired eye of *Pontellopsis regalis* (Vaissiere, 1954). Median eyes can also have mirrors to augment their ability to detect even small amounts of light. These median eyes are known as a single chambered eye with mirror.

Diversity and convergence are two key features of arthropod eyes, and of eyes in general. Many innovations in eye structure and function have been hit-upon more than once in distantly related groups (Nilsson and Osorio, 1997). In order to understand the evolution of eyes, it is necessary to combine knowledge of optics, function, morphology and especially phylogenetics.

II. EYE TYPES

A. Compound Eyes

While there are various functional types of compound eyes within the Crustacea, they share developmental and morphological characteristics. Each compound eye is a collection of nearly identical ommatidia placed in an array. Ommatidia across Crustacea possess the same cell types in similar arrangements (rev. in Melzer, 1997).

Each ommatidium comprises eight photoreceptive cells (retinular cells), four light-focusing crystalline cone cells, two corneal cells and pigment cells.

The retinular cells are long and abut one another forming a cone or cylinder. The cross-section of this cone is pie-shaped with each pie-wedge being the cross-section of a cell. The arrangements of the retinular cells and cone cells have been used as a phylogenetic signal to assign evolutionary relationships within the arthropods.

In particular, the similarities argue for the "Tetraconata", known lately as the Pancrustacea, a group including all hexapods and crustaceans. In insects and crustaceans, the retinular cell bodies tend to be arranged as three symmetric pairs (often numbered R1-6) of photoreceptive cell bodies around a central axis and the final two cells (R7 and R8) in the middle, stacked on top of one another (Melzer, Diersch, Nicastro and Smola, 1997;). Axons from all of the R cells project away from the ommatidium towards the optic lobes of the animal. The four cone cells overlie the R-cells (Figure 1).

1. Apposition Eyes

The arrangements of ommatidia in crustacean compound eyes give them various optical properties. Most crustacean groups exhibit apposition eyes. These eyes are made up of discrete ommatidia screened from each other by pigment cells. This ensures that each ommatidium only receives light entering its own lens. In this eye type, the light gathering portions of the retinular cells (termed a rhabdom) abut the lens and cornea.

Light passing through the lens goes directly to the associated rhabdom. While each lens forms its own discrete image on its rhabdom, each ommatidium is not image-forming.

All the information from the ommatidial lens is combined at the rhabdom where image data is scrambled and lost, but intensity and color (and in some cases polarization direction) are retained and sent to the brain. This information is integrated into the brain, which forms a pixelated image with each pixel representing the light information from a single ommatidium.

More ommatidia results in a higher resolution image while larger ommatidia allow for more light capture. Larger eyes seem to be one way to increase resolution, while maintaining light sensitivity. *Gammarus minus* (Amphipoda) populations in predator-rich waters have larger eyes, suggesting that the larger eyes are useful in predator avoidance (Glazier, 2011). Smaller eyes, however, are less costly energetically.

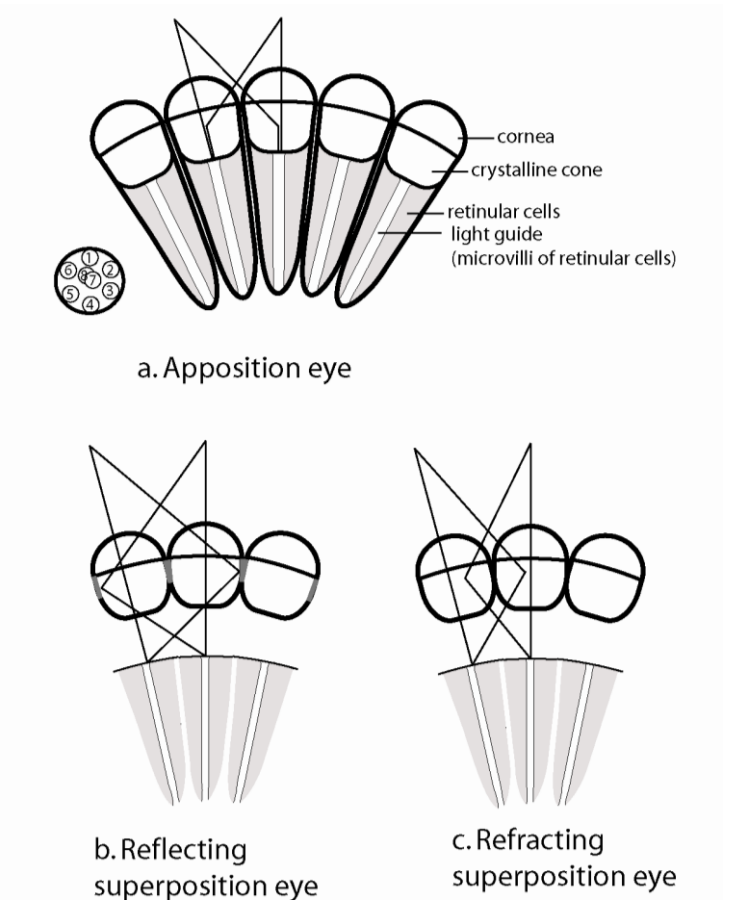

Figure 1. Apposition and superposition eyes: The typical crustacean eye consists of a dioptric apparatus (cornea and crystalline cone) overlying 8 retinular cells. The microvilli of the retinular cells are the photoreceptive surfaces. In the crustacean rhabdom, these microvilli are stacked down the central axis of the ommatidium to form a light guide. a) In an apposition eye the ommatidia are discrete units that are optically shielded from one another, often by pigment. Light (thin black lines) is focused by the cornea/crystalline cone onto its underlying rhabdom. Light focused by one lens only strikes one rhabdom. This leads to pixilated images. b and c) Superposition eyes have a clear zone between the lens and retina. This allows light from a single lens to focus on multiple rhabdomes. b) In a reflecting superposition eye, the lens acts as a mirror box (light grey), with light reflected off the sides of the crystalline cone. Note that most reflecting superposition eyes do not have a focusing cornea. c) In refracting superposition eyes, light is refracted internally by the cornea and crystalline cone onto the retina (for further explanation, see Land, 2012).

a. A Typical Apposition Eye

Apposition eyes comprise a highly variable number of ommatidia. Some crustaceans, such as some myodocopid ostracods, display fewer than a dozen per eye while other crustaceans, such as hyperiid amphipods, can have hundreds (rev. in Land and Nilsson, 2012). The ostracod *Vargula tsujii* exhibits a relatively simple apposition eye with a mere 20 ommatidia. Like other apposition eyes, each of these ommatidia is screened by pigment found primarily in accessory pigment cells (Huvard, 1990). These apposition eyes are stalked, like many other crustacean apposition eyes. This allows for scanning of the environment and increases the visual field, a task which may be especially important for an animal with such a low-resolution eye. Each ommatidium has its own dioptric apparatus made up of a crystalline cone secreted by two cone cells at the surface of the ommatidium (Huvard, 1990). Six to eight retinular cells are beneath the crystalline cone with their microvilli tightly packed along the rhabdom (Figure 1). At the proximal portion of the ommatidium, the microvillar stacks give way to the retinular cell bodies, whose axons extend into the stalk (Huvard, 1990).

b. Variations on Apposition Eyes

The typical apposition eye is a fairly homogenous structure, made up of an array of nearly identical ommatidia. Variations of this structure are common among the crustaceans, where diverse habitats and lifestyles require diverse visual information. In particular, several crustaceans exhibit a non-homogenous array of ommatidia, with some ommatidia being smaller or larger than average. These eyes correspond with the two opposing visual needs of midwater predators: to be able to clearly see small prey and to be able to detect low amounts of light. Hyperiid amphipods have solved this by splitting each compound eye in half (Land, 1989). These two portions differ in their facet size (larger facets let in more light) as well as their inter-ommatidial angles (smaller angles result in higher resolution). The upward pointing portion of the hyperiid eye has smaller interommatidial angles and larger lenses, useful for detecting small objects (prey) against the bright background of downwelling light (Land, 1989). The observations leading to these conclusions regarding amphipod eyes are greatly aided by the various habitats that hyperiids inhabit. Surface hyperiids have single eyes. The difference between the upper and lower eyes in midwater hyperiids increases with depth, culminating in the deep-living *Cystisoma*, whose eye is composed entirely of the upward-pointing ommatidia (Land, 1989; rev. in Land and Nilsson, 2012).

c. An Amazing Apposition Eye

The diversity of apposition eye forms ranges from the reduced and fused eyes of *Remicaris exoculata*, a deep sea hydrothermal vent shrimp that can see the heat of a sulphur chimney (Van Dover and Chamberlain, 1989), to the large and complex stomatopod eye. Stomatopod crustaceans, or mantis shrimp, have remarkable eyes that are able to view not only the two "typical" light modalities - color and intensity - but also linearly and circularly polarized light. Stomatopods have typical crustacean ommatidia arranged in an apposition eye pattern, but variations in ommatidial shape and properties vary greatly across the compound eye (Marshall, 1988). In particular, stomatopods have a midband, a horizontal stripe of enlarged ommatidia with high sensitivity to color and polarized light. These ommatidia in the stomatopod eye have color filters that allow individual stomatopods to "tune" their spectral sensitivity (Cronin, 2001), though it has been suggested that the stomatopod lacks the neural processing necessary to distinguish these colors (Thoen, 2012). Besides the midband, other ommatidia also vary in size across the eye and different regions of the eye have different resolving power (Marshall, 1993).

Others ommatidia detect polarized light via precisely aligned stacked microvilli in the photoreceptors, which allow the detection of polarized light (Marshall, 1998; Marshall, 1991). Circularly polarized light is detected through the stacking of photoreceptor microvilli at angles to one another. Like the typical crustacean ommatidium, stomatopod ommatidia have their R8 cell-body stacked on top of the other retinular cells. In specialized ommatidia, this R8 cell's microvilli are rotated orthogonal to the other retinular cells' microvilli. These retard circularly polarized light photons and convert them to linearly polarized light, which can be detected by the underlying retinular cells (Chiou, 2008).

Detection of these additional light modalities allows for an extremely nuanced view of the visual world. Background light, the air/water interface, and crustacean cuticles can all polarize light. Being able to detect this enhances the contrast with which stomatopods see their world. In addition, stomatopod (and other crustacean) cuticles can produce circularly polarized light reflections. Detection of this type of light is hypothesized to be involved in sexual signaling in stomatopods (Chiou, 2008).

2. Superposition Eyes

The other main type of crustacean compound eye is the superposition eye. This eye type is only found in the Eumalacostraca, a group containing shrimp

and lobsters (Nilsson, 1983). The eumalacostracans, especially the decapods, show an incredible diversity of superposition eyes. They include reflecting superposition, refracting superposition, and the rare parabolic superposition types. All of these eyes share the common feature of a gap between the lens and the retinular cells. Because of this, each rhabdom receives light information from the lenses of several ommatidia. This gives a wider field of vision for each rhabdom, compared to the apposition eye. In this way, each rhabdom is exposed to more light, a clear advantage in light-deprived marine environments. However, this leads to the problem of having each lens focus light on multiple parts of the retina. Eumalacostracans have come up with several optical solutions to this.

a. Reflecting Superposition Eyes

The most common superposition eye is the reflecting type (Porter and Cronin, 2009), and it is considered to be the ancestral eye type of the decapod crustaceans (Land, 2012). Like its name suggests, its optics are based on the principle of reflection with a biological mirror (or "prism") focusing light into the retina. Because the light is focused by the mirror, many superposition eyes do not focus light into the retina with their cornea, though there is evidence that at least one species does (e.g. Vogt, 1980; Land, 1978; Bryceson, 1981). In a typical reflecting superposition eye, the crystalline cone has a rectangular cross-section and forms a "mirror box" that reflects light off its sides (Vogt, 1975; Land 1976; Vogt, 1977). Because all four sides of the cone is reflective, rays entering the mirror box always exit parallel to their original direction (see Land, 2012 for details).

b. Refracting Superposition Eyes

More rare is the refracting superposition eye found in insects and some decapods. These eyes use a lens to focus the light. Unlike apposition eyes, which focus light onto an abutting rhabdom, refracting superposition eyes need to focus light across a clear zone onto a retina. Apposition lenses focus parallel light beams onto a single rhabdom. Superposition ommatidia focus onto multiple rhabdoms. Superposition eyes solve this problem by redirecting the focused light via cone cells with a graded refractive index (Exner, 1891; Kunze, 1979). The output of this type of eye looks similar to the output of a reflecting superposition eye as both have a similar redirection of light rays, although the reflecting superposition eye lacks a focusing lens.

c. Parabolic Superposition Eyes

Parabolic superposition eyes use parabolic mirrors combined with elongated light guides and lenses to focus light. As in an apposition eye, light enters a corneal lens and is focused onto an underlying crystalline cone, which acts as a light guide. Some light rays pass through this cone onto the rhabdom. Rays coming in at an oblique angle are reflected off the parabolic mirror sides of the cone, exiting the cone in parallel in a similar fashion to reflecting superposition ommatidia.

d. Variations on Superposition Eyes

Superposition eyes are typically spherical structures with homogenous ommatidia, like the simplest types of apposition eyes. There are fewer variations on this eye morphology compared to apposition eyes since the ommatidium is not a discrete unit in superposition eyes. The shared optics makes it difficult to produce ommatidia of different sizes and visual properties as seen in stomatopods (Nilsson and Land, 2012). Despite this, a few malacostracans have developed some amount on non-homogeneity in their ommatidia. Like other midwater crustaceans mentioned above, euphausiids can also exhibit double eyes. Since their optical components are shared across their superposition eyes, each of the eyes in the doublet is separated optically, each retina has its own set of lenses.The upward pointing eye has a smaller field of view and smaller inter-ommatidial angles (and this higher resolution) than the downward point eye. In most double-eyed species, the upper and lower eyes are similar in size. However, this does not need to be the case as selection can act on the size of the two eyes independently and can reduce the size of the upper or lower eye (Land, 2000).

e. An Amazing Superposition Eye

Taking the double eye to the extreme, *Dyoptris pacuinispinosa* are mysids with a single enlarged ommatidium underlying each of their lateral refractive superposition eyes. This enlarged ommatidium takes advantage of the separation of photoreceptive surface from lens in superposition eyes. Instead of the typical 7 or 8 retinular cells in a rhabdom associated with a single dioptric apparatus, the enlarged facet is associated with 120 densely packed rhabdoms. These thin rhabdoms have greatly reduced pigmentation (Nilsson, 1994). The facet itself is about three times the size of the facets in the rest of the eye and overlies two giant crystalline cones that have apparent functionality in focusing and directing light. A few other small cones are also found in this region, but they are unlikely to be functional. Developmental data

suggests that the large facet is made early in development, but the mechanism for switching eye development modes is unclear (Nilsson, 1994).

B. Median Eyes

Median, or frontal, eyes are simple non-image forming eyes located at the dorsal anterior midline of arthropods (Paulus, 1972). In naupliar larval crustaeans, these are the only eyes (though zoea larvae have compound eyes). These eyes are also found in adult crustaceans, often in addition to compound lateral eyes. In adults, they may be used for vision or for the entrainment of circadian rhythms (Elofsson, 2006).

Crustacean median eyes project axons to the dorsal protocerebrum. Many crustaceans have a tripartite median eye made up of three retinal surfaces (four in phyllopods) - two lateral and one ventral. These surfaces are cup-like and made up of just a few photoreceptive cells (Elofsson, 2006; Reimann, 2007). This tripartite eye is also called the naupliar eye. The term “naupliar eye” also encompasses tripartite eyes combined with an additional pair of dorsal frontal eyes, making an eye with five retina (Elofsson, 2006). While not present in most species, paired ventral frontal eyes are also possible (Elofsson, 2006). These multiple eye types can all exist together, can be entirely absent, or can be found in combination (Elofsson,1966; Elfosson, 1963).

a. A Typical Median Eye

Theostracans, branchiurans, copepods, and ostracods usually have three median eyes. In ostracods this eye is typically tri-partite while in the other groups, the three eyes are separated. (Elofsson, 2006). The barnacle *Balanus* (Theostraca) has 13 sensory cells in its dispersed median eyes (Fahrenback, 1965). These eyes comprise two pigmented lateral eyes and a ventral eye, each of which is innervated seperately. Tapetal (mirror) cells are present in the lateral eyes only (Fahrenback, 1965). Interestingly, examination of late larval *Balanus* species reveal a tripartite median eye, rather than the dispersed eyes seen in the adult (Kauri, 1962). This eye lacks tapetal cells, but does have pigmentation in two of the three eye-cups. Like the adult eye, each of the cups sends its own neve to the CNS (Kauri, 1962; Takenaka, 1993). Later studies examined shadow responses, visual pigment, and neurotransmitters, making this a particularly well-studied median eye (rev. in Elofsson, 2006).

b. An Amazing Median Eye

Although median eyes do not form images, they can still be useful detectors of light and can even sense direction of light. While the deep sea ostracod *Gigantocypris* has the typical tri-partite median eye, at first glance it appears to have instead two giant "headlamps" that take up 20% of the animals length (Land, 1978). In *Gigantocypris*, two of these three eyes are greatly enlarged and contain parabolic mirrors to enhance their ability to detect the tiniest amount of bioluminescent light available in the deep. Land (1984) estimated that their ability to collect light is 64 times that of a dark-adapted human eye. Since the mirrors in these eyes are parabolic, the curvature along their surface varies. This curvature change focuses the light into a line. This would lead to an astigmatic image on a typical retina (Land, 1984; Land and Nilsson, 2002). To partially compensate for this, the photoreceptors on the retina are elongated in the direction of the image created by the reflectors. However, since the main function of these eyes is likely simply to gather as much light as possible, any information produced above direction and intensity is probably lost in higher level processing. (Land and Nilsson, 2012).

III. EYE DEVELOPMENT

Both lateral and median eyes develop from the head ectoderm and are innervated by the protocerebrum (Paulus, 2000). Despite developing from a single embryonic anlagen in at least some cases (Elofsson, 1969), the developmental timing and the specifics of innervation are distinct enough between these two eyes to provide evidence that they evolved separately (Paulus, 2000).

A. Median Eye Development

The development of median eyes typically occurs during embryogenesis, newly hatched larvae and juveniles have median eyes, with the exception of peneid shrimp (Dahl, 1959; Dobkin, 1961; Elofsson, 1966). These early median eyes can undergo further development throughout larval and juvenile stages with the addition or degradation of photoreceptors, separation of the retinal surfaces from a single anlagen, retraction towards the brain, fusion of separated retinas, growth of tapeta, and asymmetric growth (rev. in Elofsson, 2006).

Early median eye development had been recorded from very few crustaceans. Where it has been examined, the eye anlage is a U or V-shaped structure in the developing brain. The legs give rise to compound eyes (and other brain structures in the case of *Artemia salina*) and the base gives rise to the median eyes (Dobkin, 1961; Benesch, 1969; rev. in Elofsson, 2006). The different parts of this single anlagen may then proliferate at different times such that the median and lateral eyes do not develop simultaneously (Wildt, 2002). This single developmental origin has led some authors to surmise a common evolutionary origin of all three eyes (Oakley, 2007).

B. Compound Eye Development

While some crustaceans emerge from embryogenesis as juveniles with a complete set of adult-type eyes and appendages (e.g. Wakayama, 2007), others first undergo larval development as a nauplius or zoea. Larval eyes can differ dramatically in both structure and function from adult eyes. In the case of larval compound eyes, they can undergo a change in their optics, though they likely develop using the same genetic components. Larval eyes can also be unpaired median eyes, in which case lateral compound eyes develop later from larvally retained eye anlagen.

Compound eye development has been studied in a number of crustaceans to some extent. In these cases, the eyes develop from an eye anlagen. This anlagen may arise from the lateral portions of the primordial eye anlagen (see above). Once an eye field is specified, there are still two main hurdles to creating an eye, or indeed any type of body part. Cells must be recruited or born into the field and the early anlagen must differentiate and organize into the different cell types in the correct orientation to one another. In malacostracans, a proliferation zone at the rim of the retina provides new cells for the developing eye (Elofsson, 1969; Elofsson and Dahl, 1970; Hafter, 1982; Hafner and Tokarski, 1998, 2001; Harzsch and Dawirs, 2006; Harszch 1999; Harzsch and Walossek, 2001; Wildt and Harzsch, 2002).

Crustacean compound eyes tend to develop from distal-most to proximal regions of the eye with a "morphogenic front" sweeping across the undeveloped eye field, leaving newly minted ommatidia in its wake. In malacostracan crustaceans and *Triops* (a branchiopod), new eye cells are generated in the proliferation zone and then "recruited" to form pre-ommatidial clusters of cells. These cells subsequently differentiate into mature ommatidia (Hafner and Tokarski, 1982, 1988; Harzsch and Dawirs, 1994;

Harzsch and Walossek, 2001; Wildt and Harzsch; Melzer, 2000). In crayfish this differentiation includes a sorting step wherein the retinular cells sink under the rapidly growing crystalline cone and corneagenous cells. In this way the strata of the ommatidium are established (Hafner and Tokarski, 1998).

C. Eye Remodeling

The visual needs of planktonic larvae may be very different from the diverse needs of adult crustaceans, which live in a wide variety of light regimes and occupy many ecological niches. The development of adult eyes from larval eyes can be as simple as adding additional ommatida to the eye margins or as complex as a nearly complete remodeling of the eye, such as converting an apposition to a superposition eye (Meyer-Rochow, 1975). This complete remodeling occurs in decapod crustaceans. In these animals the eyes gradually convert their ommatidia to the more complex adult type. For example in oplophorid shrimp, facet shapes in the juveniles change from hexagonal (apposition) to square (superposition) during metamorphosis. These changes begin at either the lateral or anterior portion of the eye depending on the species and progress over the eye, with the dorsal-most portion the last to change (Gaten and Herring, 1995).

Eye remodeling has been studied in some detail in mysid and euphausiid larvae (Malacostraca). In the euphausiid *Thysaoessa*, the clear apposition eye of the larvae begins to metamorphose just before the animal is released from the marsupium. In this species, ommatidial elongation is followed by the separation of the crystalline cone and the rhabdom to create a space between the lens and the retina, as in a superposition eye. As the crystalline cone moves closer to the cuticle, it begins to change to the square shape of a superposition facet. While this is occurring, pigment granules move around the crystalline cones, resulting in a darker eye. Eye transformation completes with the cone and rhabdom separating even further. The space between them is filled with unpigmented retinular cells and the agranular portion of pigment cells. Post-metamorphosis, new ommatidia are added to the outer rim of the eye from a proliferation zone (Nilsson, 1986). Eye remodeling has also been examined in the mysid *Gnathophausia ingens*. This crustacean begins its larval life in shallow water and migrates to deeper water as it matures. During this migration, the eye goes from an apposition to superposition type in much the same way as *Thysaoessa* with a gradual increase in the amount of clear space between the lens and the retina. In *Gnathophausia*, however, there is a

decrease in pigmentation as eyes mature and the animals move to a darker habitat (Whitehill, 2009).

D. Invisible Eyes

A transparent material allows light to pass through it. To act as an eye, a tissue must absorb light and, thus, be pigmented. Pigmentation can be maladaptive in small planktonic animals as it is a visual signal to their predators. This is especially relevant for larval forms, as they do not need to be visually recognized by mates but often spend much time in the water column dispersing before metamorphosis. Some larval crustaceans have solved this problem by reducing their eyes almost entirely. Others have retained eyes but altered their optics such that the pigmented area is greatly reduced. In the almost completely invisible eye of euphausiid amphipods larvae, the need for screening pigment is circumvented by crystalline cones, which optically isolate each ommatidia. They do this by being denser than normal with a refractive index gradient at the proximal ends that prevents great amounts of light from traveling between the ommatidial rhabdomeres (Nilsson, Hallberg, 1986).

In the adults, pigmentation increases greatly in the transparent eyes to become typical apposition or superposition eyes. One exception to this is the wedding shrimp, *Spongicoloides koehleri.* These shrimp colonize sponges and live in dark conditions. Instead of using mirrors and superposition optics to increase the amount of light on the retina, these shrimp retain the larval transparent apposition eyes. The lack of pigment allows more light into the eye, but this is at the expense of resolution. Thus it is thought that these invisible adult eyes prioritize higher light sensitivity without the metabolic expense of eye-remodeling or production of pigment granules (Gaten, 2007).

E. Connecting to the Central Nervous System

Photoreceptor cells in each ommatidium project axons to either the first optic neuropil (the lamina) or the second optic neuropil (the medulla). The first group are said to have Short Visual Fibers (SVFs) and the second Long Visual Fibers (LVFs). SVF cells make up the majority of photoreceptor cells in an ommatidium and are typically green light sensitive. LVF cells are sensitive to

shorter wavelength (blue and UV light) (Meinertzhagen,1991). SVF cells can also differentiate light polarization (Kleinlogel and Marshall, 2005).

Most crustaceans have a straighforward projection of axons from the retina, through the visual ganglia, to the brain. In these crustaceans, axons project from the photoreceptors in the retina to the lamina or medulla. Lamina axons project to the medulla and medulla axons project to the brain (rev. in Nilsson and Osario). This heirarchical structure is also found in malacostracan crustaceans, but here the picture is complicated by the presence of optic chiasmata. Chiasmata are crossing over points where fibers from the left hand side cross over fibers from the right hand side. In this way, projections from the left side of the retina can find themselves projecting to the right side of a visual ganglion. There are two crossing over points in malacostracans, the first is between the lamina and medulla, where LVF axons from the right visual field cross over to the left side of the medulla and LVF axons from the left visual field cross to the right. A similar crossing over creates the second chiasma between the medulla and a third visual neuropil, the lobula, found only in malacostracans and insects. In non-malacostracan crustaceans, LVF axon project straight to the medulla without crossing over (rev. in Nilsson and Osorio; Harzsch, 2002).

Developmentally, the first chiasm comes about due to the direction of axon growth between the lamina and medulla. In the insects and malacostracans, the lamina and medulla grow perpedicular to one another, leading to crossing over of axons between them. In other crustaceans, the lamina and medulla grow parallel to one another, so no crossing over occurs (Harzsch, 2002). This suggests that either the malacostracans and hexapods (the group containing insects) are phylogenetically sister groups (e.g. that the insects evolved from malacostracan crustaceans) or that chiasmata and the lobula evolved more than once from the more basic non-malacostracan crustacean-type system. Since it seems unlikely that the malacostracans and hexapods are sister taxa (Regier, 2010), the second hypothesis is more probable. A third scenario is envisioned by Nilsson and Osario (1997) in which the pancrustacean/hexapod ancestor lacked compound eyes and had only simple ocelli connected to a single optic ganglion. In the insect and malacostracan lineages, these evolved into the three-neuropil system with two chiasmata while in the non-malacostracans, these evolved in the the two neuropil system. This hypothesis, however, is predicated on the notion that the mutations involved in going from the simpler system to the two chiasmata system would be complex and unstable. The later developmental data by Harzsch (2002) suggests a fairly simple developmental mechanism for

generating the more complex system. Thus it seems likely that the two chiasmata system evolved twice from a simple two neuropil system. This finding demonstrates the point made by Gaten (1998) on the dangers of creating phylogenetic inferences based on eye morphology. While complex eyes may be an attractive source of phylogenetic information, the signal may be lost due to high levels of convergence as adaptive pressures act on distantly related organisms inhabiting similar specialized environments (Gaten, 1998).

IV. GENETICS

The study of crustacean eye genetics is still in its infancy. While transcriptomes of a few model crustaceans have been sequenced, only a single crustacean genome - that of the water flea *Daphnia pulex* - is publicly available. Of the sequenced transcriptomes, few have published data associated with them regarding developmental genes. Several single-gene studies have been published regarding the genetics of specific crustaceans. These have generated candidate genes for future studies comparing the expression patterns and functions of eye-genes across crustacea. Because of this and because of the plethora of developmental and genetic data available from several insects, members of the Pancrustacea, the study of crustacean eye genetics lacks only higher-throughput techniques for examining multiple genes across several species. Especially interesting will be future studies comparing the development of the various eye-types, especially the metamorphosis from apposition larval eyes to superposition adult eyes in the malacostraca.

As one of the original developmental genetic model systems, the fruit fly *Drosophila* has much to add to our understanding of crustacean eye genetics. While *Drosophila* compound eyes exhibit highly derived optics (neural superposition) they also exhibit many similarities with crustacean eyes. For example *Drosophila* have simple median eyes and compound larval eyes. Importantly, *Drosophila* eye development follows the same pattern as crustaceans including similar proliferation patterns, growth of optic lobes, retinal pattern formation, and ommatidial differentiation (Harzsch, 2002). In this way, *Drosophila* eyes are likely a highly useful starting point for understanding the evolution of crustacean eye genetics.

Comparisons of eye development between *Drosophila* and *Tribolium* as well as between the different *Drosophila* eye types have yielded a long list of genes important in eye development and phototransduction (Bao and Friedrich, 2009). The developmental genes are thought to act in a hierarchy

with increased specification as development proceeds. The early genes are pro-neural and specify eye-tissue. These include some paired-box (Pax) genes and their network partners Dacshund (Dac) and Six as well as the downstream gene Hedgehog. Later genes are involved in differentiation of the eye field and include Glass, Runt, Lozenge, Orthodentical, Tramtrack, Homothorax, Elav, and Chaoptic. Once eyes have been established, phototransduction proteins are responsible for recognizing light (Opsin) and transducing the signal to the nervous system. Other phototransduction genes found in crustaceans include Phospolipase C (PLC, aka norpA), retinal degeneration C, Calmodulin (Cam), and Calx. Other eye-genes have been annotated in the *Daphnia* genome, but have not yet been found in other crustaceans and the expression pattern and function have not been determined.

A. Gene Duplication

The success of the crustaceans to adapt to and thrive in diverse light environments may be driven by this lineage's history of generating wildly diverse eye types. How this lineage, of all others generated this enormous morphological diversity is an open question. The evolution of the compound eye itself is unlikely to be the driver as this eye-type has evolved in other lineages without the diversification seen in the pancrustaceans (Land and Nilsson, 2012). Since gene duplication, followed by retention and divergence, is thought to be one of the primary drivers of the evolution of novelty could this be a factor in crustacean eye evolution? Of about 20 genes involved in eye development or phototransduction, several show higher levels of duplication and retention in pancrustacean lineages than in other animal lineages (Rivera, 2010). In particular, several genes involved in photoreceptor differentiation duplicated at the base of the pancrustacean tree. Other duplications were specific to the insect lineage. Opsins underwent massive amounts of duplication during pancrustacean evolutionary history, a finding which has not yet been resolved with respect to photoreceptor sensitivity in this group (Rivera, 2010).

Unfortunately, this type of analysis depends on robust, high coverage genome sequences in the organisms to be tested. Since there is only one crustacean genome currently publicly available (Colbourne, 2011), this dataset relied largely on insect genomic data. As more crustacean genomes and EST libraries become available, this type of analysis can be repeated on a much

larger scale to look for particular gene families and gene types that may be correlated with crustacean eye diversification.

B. Determination Genes

1. Drosophila

Eye determination genes act early in eye development to specify the eye field, overexpression of some, including Toy, Ey, and Optix, can induce ectopic eyes. In *Drosophila*, these include, among other genes, the Pax genes Eyeless (Ey) and Twin-of-eyeless (Toy), Eyegone (Eyg) and Twin-of-eyegone (Toe). Eyeless and Toy are expressed throughout embryo development. Eyeless acts downstream of Toy, along with several transcription factors and nuclear proteins, in the determination steps of eye development. Together, Eyeless and Toy regulate compound eye development, whereas Toy alone regulates ocellar development (Aspiras, 2012). Eyg works cooperatively with Ey to promote eye development in addition to inducing the expression of Dachshund (Dac) and Optix; two genes also important in eye determination. Additionally, Dac may also induce ectopic formation of the retina. Dac normally functions as a positive regulator of Eyes absent (Eya) (Shen, 1997; Chen, 1997). Decapentaplegic (Dpp) and Hedgehog (Hh) also are Eya inducers. In particular, Dpp functions to stimulate eye development through antagonizing the expression of Wingless (Wg), which would otherwise function to inhibit eye development and promote head capsule development. Hh expression plays a role in the formation of photoreceptor cells and the control of the morphogenetic furrow. It also plays a role in patterning and ommatidial assembly (Dominguez, 1997). Engrailed (en) is a homeobox transcription factor known to distinguish the posterior and anterior compartments in each segment of a developing animal. En mutants exhibit highly variable phenotypes (Brower, 1986). A second homeobox gene involved in eye-field determination is Lim1. Lim1 acts with another LIM-homeodomain protein, Arrowhead, to prevent retinal differentiation in regions of the eye disc destined to become ventral head tissue, thus avoiding ectopic expression (Roignant, 2010).

2. Crustaceans

While the genes described above have all been sequenced in at least one crustacean, few have associated functional or expression data. Eyg and Eya are known only from *Daphnia* genome traces, Ey and Toy have only been

sequenced in two crustaceans, *Daphnia* and *Euphilomedes carcharodonta* (Ostracoda) and expression data has not yet been published (Colbourne, 2011; Rivera, 2010). Hh function has been deduced by comparing cave- and non-cave-dwelling amphipods within and between species. In both *Styanax mexicanus* and *Gammarus minus*, Hh levels were reduced in the cave-dwelling species, while other eye determination genes like Pax6 (Ey and Toy), Sine oculis, and Dachshund, were not reduced. This suggests that Hh may be a consistent target of evolution in the adaptation to cave environments (Aspiras, 2012). Another species with both cave and non-cave morphotypes is *Asellus aquaticus.* Protas et al (2011) examined genetic loci linked to eye loss using QTL analyses. Between the surface and cave-dwelling species, there are clear differences in Lim1, or a closely linked gene. These differences are correlated with eye loss in the cave-dwelling species, though the exact gene responsible for the phenotype is as yet unknown.

Many determination genes are known to have multiple roles in development. En, for example, is also involved in setting up segments along the anteroposterior axis of arthropod embryos, including crustaceans and has been sequenced in a number of crustaceans including *Artemia franciscana*, *Procambarus clarkii*, *Porcellio scaber*, and *Asellus aquaticus* (Manzanares, 1993; Ahzhanov, 2000; Scholtz, 1994; *A. aquaticus* sequenced unpublished, NCBI accession ADG96401). At least one study also found En expressed in the eye of an amphipod (Scholtz, 1994). Dac, a gene involved in both retinal and appendage development in *Drosophila*, has only been studied in crustacean legs (Sewell, 2006). A handful of other eye determination genes from various crustaceans have been sequenced, but their role in eyes has not yet been determined. These include the Notch family genes of *Artemia sinica* (NCBI accession ADP89476), *Daphnia magna* (NCBI accession AEO91999), *Parhyale hawaiensis* (NCBI accession ABK56706), *Panulirus argus* (NCBI accession ACT64632), and *Daphnia pulex* (Rivera, 2010; Colbourne, 2011), Optix from *Parhyale* (NCBI accession EU908055) and Lim1 from *D. magna* (NCBI accession AB539165).

C. Differentiation Genes

1. Drosophila

During ommatidial morphogenesis within *Drosophila*, photoreceptors differentiate in the posterior to anterior direction, known as a morphogenetic furrow. This furrow is created due to the contraction between differentiated

and undifferentiated cells. Wg is required for the proper patterning of the retina during this stage of morphogenesis. Its expression functions to regulate the differentiation of the eye discs into adult head tissue and to keep the furrow aligned through inhibition of furrow progression. Wg also functions in the dorsal and ventral patterning of the eye disc.Ectopic expression and mutations in wg demonstrate its functionality. When Wg is expressed ectopically in the center of the eye disc, where contraction of the furrow normally occurs, it blocks the progression of the furrow (Wolff and Ready, 1993). Loss of function of Wg shows morphological changes, in which the head is misshapen, and the furrow begins in the lateral margins of the eyes, as opposed to the center of the eye disc. Ectopic expression further demonstrates a role for Wg in furrow position and initiation. (Duman-Scheel, 2002). Embryonic lethal abnormal vision (Elav) is another early differntiation gene essential for normal development of optic lobes and eye discs. (Campos, 1985).

Eye-cell differentiation involves a number of genes including Homothorax, an early negative regulator of eye development which later determines the cell fate in the inner photoreceptor cells of eyes, inducing the polarisation sensitive dorsal rim area fate instead of the default color sensitive fate (Pai, 1998. Wernet, 2003). Chaoptic (chp) is associated with rhabdom organization, as chp mutants have reduced, or entirely absent, rhabdoms (Pollock, 1990). A final gene involved in photoreceptor differentiation is Ocelliless, also known as orthodenticle (Oc). It is a target gene of Hh during early eye-antennal disc development, and is involved in the regional segregation of Hh and Wg (Van, 1993. Royet, 1996).

2. Crustaceans

Most eye differentiation genes in crustaceans are known from sequence data only Senseless, Oc, EGFR, Vsx, and Glass have only been identified on the sequence level in the *Daphnia* genome (Rivera, 2010; Colbourne, 2011). Glued, Tramtrack, Elav, and Chaoptic are known only from sequence data in crustaceans (NCBI accessions ADD38365, JP328060 JP319382, BT078295).

A wg ortholog in *Mysidium columbiae* (mcoWg) is expressed during mysid eye development. Its expression is detected solely in the dorsal region of developing segments and within the developing eye; unlike *Drosophila* wg, it is not expressed in the ventral neuroectoderm. Retinal patterning in the development of both mysids and *Drosophila* is conserved, suggesting that McoWg and wg function are also conserved. (Dunam-Scheel, 2002). Like Dac and En, some differentiation genes have primarily been studied in their role in other developmental contexts. Homothorax plays a role in the patterning of

Parhyale hawaiensis legs; however, it is still unknown as to what role Hth plays in eye development (Pripic, 2008).

D. Phototransduction Genes

1. Drosophila

The phototransduction cascade begins with the activation of Opsin. Opsin is a transmembrane receptor that uses a small molecule called retinal, derived from Vitamin A, to change conformation when a photon of light hits it. Opsin interacts with other proteins through a signal transduction pathway to ultimately excite its photoreceptor cell (see Plachetzki, 2010). The phototransduction cascade relies on robust termination and deactivation factors to quickly stop signalling upon cessation of stimuli. Retinal degeneration C (rdgC) is a calmodulin-dependent protein phosphatase that plays a role in the dephosphorylation of rhodopsin and the termination of the photoresponse (Lee, 2001). NorpA also plays a role in the termination of the light response in the retina after cessation of the light signal. NorpA mutants are blind and have reduced number photoreceptorin their compound eyes and ocelli (Wang, 2008; Yoshioka, 1983). Cam plays a role in the termination of the light response through controlling receptor and ion channel activity in photoreceptor cells (Scott, 1997). Calx excanges calcium and sodium across membranes, it plays a role in the prevention of retinal degredation through excess Ca2+, by modulating Ca2+ levels in the retina (Wang, 2005). Arrestin contributes to the arrest of the phototransduction cascade via binding to rhodopsin (Yasuike, 2010).

2. Crustaceans

Like all animals with eyes, the crustaceans use Opsin as their primary photoreceptive molecule. A visual pigment comprises a specific Opsin and its associated retinal molecule. Multiple types of visual pigments (i.e. multiple opsin genes) are found in crustaceans. Many of these are able to respond to different light wavelengths. Most crustaceans are di or tri chromatic, but some species can detect many more colors. For example, stomatopods have up to 16 different visual pigments and *Daphnia*, a branchiopod, has 46 opsins (the primary component of visual pigments) (Porter, 2009; Colbourne, 2011). Interestingly, *Daphnia* has only 22 ommatidia and is only known to have 4-color vision, plus the ability to detect polarized light. Microarray data suggest

that at least some of the more similar opsins are expressed in overlapping but unique patterns over the course of the animal's life (Colbourne, 2011).

Non-Opsin phototransduction genes are less well studied in crustaceans. NorpA is known only from the *Daphnia* genome (Colbourne, 2011). Arrestin is known to be expressed in *Triops* eggs, but its role in phototransduction is untested (Kashiyama, 2010). Other genes including Calx and Cam are known only from their function in other processes in crustaceans (Ziegler, 2002; Vanschoenwinke, 2012; Ji, 2011).

V. EVOLUTION OF EYES

Compound eyes had evolved by the Cambrian, they are found in stem group crustaceans as well as chelicerates (horseshoe crabs) and trilobites. Typically they are thought to have evolved from a collection of single eyes, like the ocelli of myriapods, that gradually came together and fused (Bitsch and Bitsch, 2005). The evolutionary path to compound eyes, however, is largely unknown. The recent finding of a well-preserved anomalocarid eye has confused matters further. *Anomalocaris* were early Cambrian predators with stalked eyes. Most fossils lack eye detail but a few isolated eyes exhibit hexagonal facets similar to trilobite, chelicerate, and crustacean compound eyes (Paterson, 2011). These eyes potentially had immensely good resolution; the fossilized portions have over 16,000 facets (corresponding to ommatidia) each (Paterson, 2011). Despite having crustacean-like eyes, *Anomalocaris* is not considered to be a crustacean but rather a member of the Radiodonta, a stem-group arthropod taxon well outside extant arthropod taxa (Kuhl, 2009). This pushes the evolution of compound eyes to before the emergence of the Euarthropoda. Thus, crustacean apposition eyes may represent the basal arthropod condition, though this is contested by the traditional view that compound eyes evolved from lateral ocelli within the Euarthropoda (Melzer, 2009). This hypothesis would necessitate compound eyes evolving from simpler eyes multiple times in arthropod history. However, as ocelli are quite similar structurally to ommatidia, this is not necessarily a difficult hurdle. In fact, once lost compound eyes may be able to re-evolve, possibly by using the pleisiomorphic developmental genes and pathways used to maintain median eyes (Oakley and Cunningham, 2003; Rivera 2009). In this way the typical arthropod compound eye may have potentially evolved multiple times from ocelli precursors. One piece of evidence that may support this view is the finding of unicorneal lateral eyes in insects and crustaceans. These eyes are

primarily found in hexapods but also are seen in the downward pointing eye of the double-eyed mysid *Dioptromysis paucispinosa*. These eyes consist of a single ommatidium, though in the case of *D. paucispinosa* the retina of the uniocorneal eye is extensive consisting of 120 rhabdoms, each with its own complement of 8 retinular cells (Nilsson and Modlin, 1994). Bitsch and Bitsch (2005) argue that these unicorneal eyes represent a reversion to an ancestral-type ocellar eye.

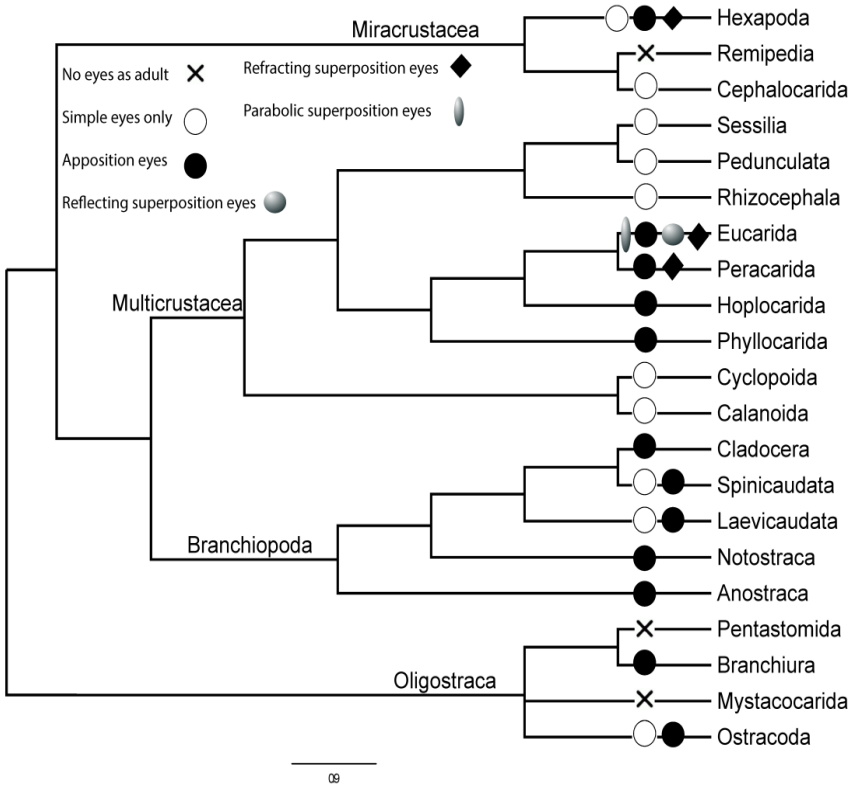

Figure 2. Distribution of optical designs in pancrustacea. Over half of all pancrustacean orders have at least some species with compound eyes. For the most part these are apposition type eyes, though in the malacostracans apposition eyes are present in the Eucarida (includes Decapoda) and Peracarida. Phylogeny from Regier et al (2010), eye characters from Porter and Cronin (2009), Land and Nilsson (2012), and Gaten (1998). A newer phylogeny (Oakley, 2012) is similar with a couple notable differences. In the Oakley et al phylogeny, Branchiopoda groups with the Miracrustacea to form a monophyletic Allotriocarida. In addition, within some of the larger groupings there are differences, for example the cirripedes (Sessilia, Pedunculata, Rhizocephala) are the sister to the copepods (Cyclopoida, Calanoida) in the Oakley tree. The larger group to which these belong, the Multicrustacea, has the same members in both trees.

Although the strict homology of compound eyes remains uncertain, similarities between ommatidia in various crustacean and insect groups argue for homology of the ommatidium across Pancrustacea (Paulus, 2000; Melzer, 1997).

The strongest argument of homology from morphological characteristics is the retinular and cone cell patterning of the ommatidium. Although there are numerous exceptions (see Oakley, 2003) ommatidia in the Pancrustacea tend to consist of 8 retinular cells, 4 cone cells, and 2 accessory cells (corneagenous or pigment cells).

The arrangement of these cells is also highly similar (see Figure 1). Perhaps an even stronger argument is the molecular finding of a monophyletic Pancrustacea (e.g. Boore, 1998; Giribet, 2001; Regier, 2010). Given the presence of multi-ommatidia compound eyes in nearly every major pancrustacean group (Oakley, 2003; Porter, 2008) it is extremely unparsimonious to hypothesize a pancrustacean ancestor without compound eyes.

This, however, does not rule out the possibility of loss and regain of compound eyes within the Pancrustacea. In fact, this pattern seems to be the case in the ostraocods where compound eyes were apparently lost in the lineage but regained in the Myodocopida, a large group of marine ostracods (Oakley, 2002).

A. Evolution of Superposition Eye Types

Superposition eyes are thought to have evolved multiple times from apposition eyes due to their improved optics in dim conditions (Nilsson and Osorio, 1998).

Decapods likely evolved the superposition eye early in their lineage, it is found across their phylogenetic tree. A trickier question is when and where did the various types of superposition eye evolve. The phylogenetic distribution of the eye types is complicated by the fact that decapod phylogeny is under flux (compare Porter 2005 and Toon, 2009 (in Decapod Phylogenetics)).

Moreover, since most decapod larvae have apposition eyes, it is unclear how much of the adult structures are merely derived from juvenile optics, as opposed to a de-novo evolution of an optical solution (e.g. Gaten, 2007).

B. The Fossil Record

1. Median Eyes

Although median eyes appear to be pleisiomorphic for the pancrustaceans, some crustaceans lack these organs. This is thought to be due to reduction of an ancestral character because of the presence of frontal eyes in sister groups to the taxa with reduced or missing eyes (rev. in Elofsson, 2006). The fossil record is of limited utility when assessing the evolutionary history of median eyes as, unlike external compound eyes, these eyes are often internal do not tend to fossilize well enough to determine fine structure. Nonetheless, clear median eyes are present in fossil crustaceans, including stem-group crustaceans (e.g. Muller, 1983; Siveter, 2010; Stein, 2008).

2. Compound Eyes

Crustacean fossil descriptions typically lack detailed morphological analysis of their eyes. Even when such descriptions exist, eyes are often imperfectly preserved and apposition and superposition eyes are very similar unless optical elements are examined. For these reasons, the record of fossil eyes is extremely limited (e.g. Rolfe, 1985). Of crustaceans with preserved eyes, the description is typically limited to external facet (lens) morphology (Castellani, 2012 and references therein). Since square facets are found in superposition eyes and hexagonal facets in apposition eyes, examination of facet patterns in crustacean fossil eyes could help to resolve questions regarding the evolution of superposition from apposition eyes (Gaten, 1998).

3. A Fossil Eye

Henningsmoenicaris scutula is an ancient fossil crustacean from the Cambrian. Even at this early date, compound lateral eyes are recognizable. *H. scutula* eyes are stalked and have an interesting “mace-like” appearance with their 200 ommatidia covering both the outward and inward facing portions of the eye. Like many crustaceans, *H. scutula* eyes have smaller ommatidia, and thus better resolution, in the forward facing portion. Unlike other crustaceans, these eyes have inward facing ommatidia, allowing for double coverage of their field of vision and, presumably, good prey tracking ability even in this low-resolution eye (Schoenemann, 2011). Interestingly, the *H. scutula* eyes are apparently not fully formed at the end of embryogenesis or larval stages. Instead, they seem to grow throughout their successive molts, with new ommatidia being added through juvenile stages (Castelliani, 2012)

C. Eye Reduction

Frontal simple eyes and lateral compound eyes have different functions in individual crustaceans so it is not surprising that the reduction of one does not necessarily coincide with the reduction of the other. Image quality tends to diminish with light-capturing ability so image forming eyes are not particularly useful for species in the light-starved environments of the deep sea or subterranean caves. Nilsson and Osorio (1998) suggest that reduction in lateral eyes (e.g. loss of ommatidia) can come about when there is a loss of selection for vision, for example with burrowing animals. A lack of lateral eyes can be seen in some cave species as well as in some deep sea species (e.g. Protas, 2011; Kornicker 1996). In at least some of these species, the median eye remains. Since this eye does not need any power of resolution, it can instead devote its form to photon capture. In some cases this leads to large and distinctive eyes with the ability to detect the faint bioluminescent signals that are the only light past 1000 meters (Land, 2000).

Loss of image-forming eyes is not necessarily an evolutionarily blind-alley. Several authors posit that a re-invention of optics can occur after a great reduction (Nilsson and Osorio, 1998; Oakley and Cunningham, 2002; Rivera and Oakley, 2009). The ampeliscid amphipods with their simple lateral eyes are one possible example of this. This loss and regain can be seen clearly in the myodocopid ostracod crustaceans where compound lateral eyes are found in a smattering of clades, some of which are apparently only distantly related (Kornicker eye list). Especially interesting in the myodocopid ostracods is the finding that the closest related groups of crustaceans, including other ostracod clades lack lateral eyes entirely. This has led to the hypothesis that myodocopids re-invented compound eyes (Oakley and Cunningham, 2002). The myodocopid compound eye is a standard crustacean eye with the normal complement of cells in each ommatidium (Huvard, 1990; Rivera and Oakley 2009). This suggests that the genetic information for building a compound eye was not lost, merely “turned off” for a period of ostracod evolutionary history.

Median eyes can also be reduced, sometimes without the concomitant reduction of the lateral eyes. For example, brachyurans (crabs) have reduced or absent median eyes but their compound eyes are well developed and encompass apposition, reflecting, refracting and parabolic superposition eyes (Gaten, 1998). In many species across Crustacea with reduced median eyes, these eyes are withdrawn towards the brain (Elofsson, 2006). They may still be able to detect light (e.g. Eaton and Boyd, 1970), and may possibly be involved

in circadian rhythm entrainment as in *Drosophila* (e.g. Sandeman, 1990; Frelon-Raimond, 2002; Helfrich-Forster, 2002).

CONCLUSION

Recent molecular phylogenies that cement Pancrustacea as a monophyletic clade greatly enhance our ability to understand eye evolution in this clade. They also set the stage for future experiments in eye development and genetics. In the past, most developmental studies have focused on generating data for understanding arthropod systematics. Now that at least some of these issues have been resolved, developmental studies can aim at differences between crustaceans, for example how different optical structures are built during development (e.g. Gaten, 2007). Combining genetic and developmental studies, as has been done in *Drosophila*, will be especially fruitful in understanding the differences in the diverse crustacean eye-types. Currently, the genetics and development of crustacean eyes are extremely understudied. With new robust molecular phylogenies that are not dependent on developmental characters and hand-waving arguments, it is hoped that developmental studies can now focus on developmental genetics and other evolutionary questions.

Crustaceans have an enormous amount of potential study systems for develpmental genetics and evo-devo studies. Some currently under investigation include the evolution and development of sexual dimorphism in ostracods (Rivera, 2009) and the evolution of eye-loss in cave amphipods (Porter, 2012). Future studies will focus on available EST libraries (sequences of all expressed genes) and will generate new EST libraries. Cheap, fast sequencing makes this type of data readily available and new user-friendly methods of analyzing these data are becoming more powerful (e.g. Galaxy). Comparing ESTs between species, morphs, and different developmental time points will generate hypotheses regarding developmental genetics and evolution in this group. Searching EST libraries for potential genes of interest, such as differentiation and phototransduction gene candidates from *Drosophila*, can be followed by expression analysis (such as in situ or qPCR) or even functional analysis via RNA interference.

These new techniques are likely to add greatly to our understanding of crustacean eyes. More traditional techniques can also be used to great effect in future studies. For example, many interesting crustacean eyes have not yet been analyzed at the microstructural level or had their optical properties

examined. These types of studies may uncover even more novelty, or perhaps more similarity, in crustacean eyes. They will also allow us to hypothesize the ecological role of these eyes for the animals studied, for example: are they involved in mating? predation? migration? Studies of fossilized eyes are also becoming more prevalent in the literature (e.g. Castelliani, 2012). While the structure of retinal tissue is not preserved, observations on the shape of the facets in a phylogenetic context may help determine whether the eye is apposition, refracting or reflecting superposition type.

Clearly, disparate fields have much to learn by further study of crustacean eyes. Comparisons of eye morphologies and developmental programs can give rise to or test evolutionary hypotheses. Ultrastructural studies can uncover new optical designs and materials useful for biomedical and biomaterials research. Studies of fossils offer a window into the past for examining early diversification. New sequencing techniques and phylogenetic hypotheses allow us to hone our questions regarding this group. These, plus a long tradition of careful morphological analyses, will give rise to the next generation of crustacean eye research.

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Chapter 2

IMPACT OF HIGH ENERGY IRRADIATION ON CHITIN AND CHITOSAN: A SHORT REVIEW

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ABSTRACT

Chitin is a structural polysaccharide and is the second most important natural polymer in the world. The main sources exploited are two marine crustaceans, shrimp and crabs. Chitin is a homopolymer of 1-4 linked 2-acetamido-2-deoxy-β-D-glucopyranose, although some of the glucopyranose residues are deacetylated and occur as 2-amino-2-deoxy-β -D-glucopyranose. Despite its huge availability, the utilization of chitin has been restricted by its intractability and insolubility. The fact that chitin is as an effective material for sutures essentially because of its biocompatibility, biodegradability and non-toxicity together with its antimicrobial activity and lowimmunogenicity, points to immense potential for future development. When chitin is deacetylated to more than 50% of the free amine form, it is referred to as chitosan. Chitosan has attracted considerable interest due to its biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries. In the recent years considerable research efforts

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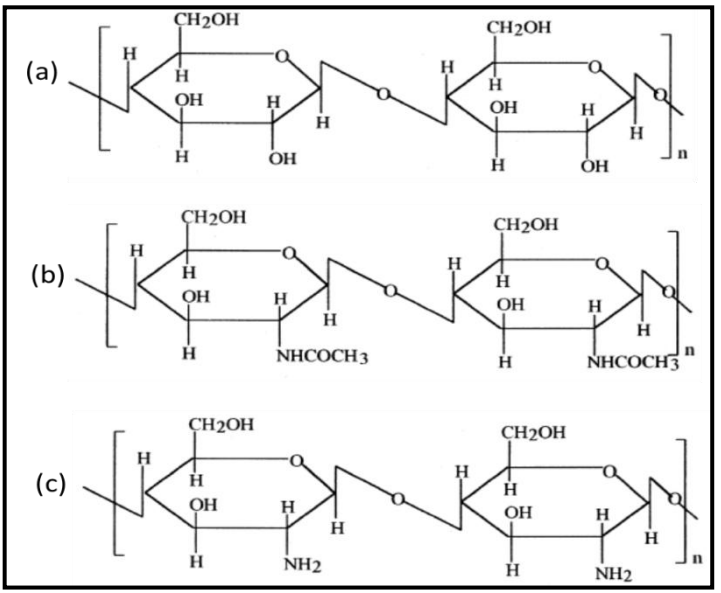
have been directed towards establishing a suitable method of extraction of chitosan and approaches to enhance its different properties. Many researchers have focused on application of gamma radiation on chitin and chitosan to obtain a potential bioactive material. This review wishes to provide an overview of the effect of gamma radiation on the extraction of chitosan from exoskeleton of crustacean (prawn shell) based on our and others' latest research results. In addition this review takes a closer look on comparison of physicochemical, thermal and morphological properties of gamma irradiated chitosan with those of the nonirradiated chitosan and potential applications of irradiated chitosan in various fields. From literature survey, it is realized that research activities on uses of gamma radiation on chitosan to improve its biomedical and agricultural applications have increased at the rapid rate. Hence, the present review is timely.

1. INTRODUCTION

Quite a few kinds of polysaccharides occur in nature in a broad range of structures and forms, and most of them are considered to work as structural materials or suppliers of water and energy, though their functions may not have been fully comprehended. Since polysaccharides have peculiar structures and properties, quite different from those of synthetic polymers, they are considered promising biopolymers for developing desirable advanced functions. Among numerous polysaccharides, cellulose and chitin are produced in the largest amounts, estimated to be around 10^{11} tons each per year, and actually, they are the most abundant organic compounds on earth. Cellulose and chitin are structurally similar to each other; chitin has an acetamido group at C-2 in place of the hydroxy group in cellulose (Scheme 1). In recent years, there have been significant advances in multidimensional uses of natural polymers due to scientist's particular interest to them. Natural polymers are biodegradable, abundantly available, and easily decomposable in the environment and eco-friendly which draw special attention of the researchers. Chitin, the second most abundant natural polymer after cellulose in nature, is one of the pioneer polymers having unique structures, multidimensional properties, highly sophisticated functions and wide ranging applications in biomedical and other industrial areas [1-3]. Being considered to be materials of great futuristic potential with immense possibilities for structural modifications to impart desired properties and functions, research and development work on chitin and its derivatives chitosan have reached a status of intense activities in many parts of the world [4-6].



Scheme 1. Structure of (a) cellulose, (b) chitin and (c) cellulose.

Despite the widespread occurrence of chitin, up to now the main commercial sources of chitin have been crab and shrimp shells. In industrial processing, chitin is extracted from crustaceans by acid treatment to dissolve calcium carbonate followed by alkaline extraction to solubilize proteins. In addition a decolorization step is often added to remove leftover pigments and obtain a colorless product. These treatments must be adapted to each chitin source, owing to differences in the ultrastructure of the initial materials (the extraction and pre-treatment of chitin are not described in this paper). The resulting chitin needs to be graded in terms of purity and color since residual protein and pigment can cause problems for further utilization, especially for biomedical products. By partial deacetylation under alkaline conditions, one obtains chitosan, which is the most important chitin derivative in terms of applications. Studies on chitin and chitosan have been intensified since 1990 because these polysaccharides show excellent biological properties such as biodegradation in the human body [7] and [8], and immunological [9] and [10], antibacterial [11] and [12], and wound-healing activity [13], [14] and [15]. In recent studies, especially, chitosan has been found to be a good candidate as a support material for gene delivery [16], cell culture [17], and tissue engineering [18,19] and [20]. Therefore, chitin and chitosan are

receiving greater attention as novel functional materials. Despite their interesting biological properties, utilization has been scarcely developed.

During the past few decades, there has been great scientific and commercial progress in processing and extraction of chitin and chitosan. Some reports the application of ionizing gamma radiation on processing and extraction of chitosan. Use of ionizing radiation not only enhances the deacetylation rate of chitin but also causes changes in physicochemical, thermal, morphological properties of chitosan through cross linking, degradation and other structural changes [21-23]. This review aims to present state-of-the-art knowledge on the impact of high energy gamma irradiation on prawn shell derived chitin and chitosan. Furthermore this will also show that the irradiation of prawn shell and how the properties of the chitosan can be altered simply by applying different doses of gamma irradiation. This will also addresses some recent development on the application of gamma irradiated chitosan in biomedical and agriculture fields.

2. STRUCTURE OF CHITIN AND CHITOSAN

Chitin possesses a highly ordered structure with an excess of crystalline regions and appears in three polymorphic forms. Depending on its source, chitin occurs as two allomorphs, namely the α and β forms [24,25], which can be differentiated by infrared and solid-state NMR spectroscopy together with X-ray diffraction. A third allomorph γ-chitin has also been described [26], but from a detailed analysis, it seems that it is just a variant of the α family [27]. α-chitin is by far the most abundant; it occurs in fungal and yeast cell walls, in krill, in lobster and crab tendons and shells, and in shrimp shells, as well as in insect cuticle. It is also found in or produced by various marine living organisms. When the degree of deacetylation of chitin reaches about 50% (depending on the origin of the polymer), it becomes soluble in aqueous acidic media and is called chitosan. The solubilization occurs by protonation of the $-NH_2$ function on the C-2 position of the D-glucosamine repeat unit, whereby the polysaccharide is converted to a polyelectrolyte in acidic media. Chitosan is the only pseudonatural cationic polymer and thus, it finds many applications that follow from its unique character (flocculants for protein recovery, depollution, etc.). Being soluble in aqueous solutions, it is largely used in different applications as solutions, gels, or films and fibers. It is chemically defined as a copolymer of two residues: 2-acetamido-2-deoxy-β-D-

glucopyranose and 2-amino-2-deoxy-β glucopyranose. The proportion of glucosamine is higher than N-acetylglucosamine, producing much better solubility in an aqueous solution of organic and numerous inorganic acids. The difference between chitin and chitosan lies in the degree of deacetylation (DD), usually defined as the ratio of the number of glucosamine to the total amount of N-acetylglucosamine and glucosamine, being the most important parameter determined for chitosan and chitin. In chitin, the degree of acetylation (DA) is typically 0.90 indicating the presence of some amino groups (as some amount of deacetylation might take place during extraction, chitin may also contain about 5-15% amino groups) [28, 29]. Chitin is a structural biopolymer, which has a role analogous to that of collagen in the higher animals and cellulose in terrestrial plants [30, 31]. Chitin may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group [32, 33]. Plants produce cellulose in their cell walls and insects and crustaceans produce chitin in their shells [34]. Cellulose and chitin are, thus, two important and structurally related polysaccharides that provide structural integrity and protection to plants and animals, respectively (as shown in Scheme 1) [35, 36]. In the solid state, chitosan is a semicrystalline polymer. Its morphology has been investigated, and many polymorphs are mentioned in the literature. Single crystals of chitosan were obtained using fully deacetylated chitin of low molecular weight [37]. The influence of experimental conditions on the crystallinity has also been described [38, 39]. The main investigations of chitosan concern its preparation with varied molecular weights and DA from chitin, the dependence of its solution properties on the DA, the preparation of derivatives and applications. Sponges, powders and fibers can be obtained by regeneration of chitosan or its derivatives from solutions.

3. EXTRACTION OF CHITIN/CHITOSAN FROM PRAWN SHELL

A variety of procedures have been found in the literature over the years for the preparation of chitin and chitosan. Main sources of the commercial chitosan come from crustaceans such as prawn, crab, krill and crawfish primarily because large amounts of the crustacean exoskeleton is available as a byproduct of food processing [40]. Crustacean shells mainly contain about 20~30% chitin on a dry basis. This proportion varies with species and with season. Thus, the method of chitin/chitosan preparation can vary with different

sources. Isolation of chitosan from crustacean shell wastes consists of four basic steps including deproteinization (DP) for protein separation, demineralization (DM) for calcium carbonate separation, decoloration (DC) for pigments separation, and deacetylation (DA) for removal of acetyl groups. Chitin can be isolated from crustacean shell wastes by two basic steps; deproteinization and demineralization which involves the dissolution of calcium carbonate with 1.0 N HCl and the removal of proteins with 3% NaOH, respectively. These two steps can be conducted in a reverse order [41]. In third stage, pigments, melanin and carotenoids can be eliminated with 0.02% potassium permanganate at 60°C, or sodium metabisulfite, hydrogen peroxide or sulfuric acid. During deacetylation, conditions must sufficiently deacetylate the chitin to yield a final chitosan product that is soluble in dilute acid solution. It is generally achieved by treatment with concentrated sodium hydroxide solution (40~50%) at 100°C or higher temperature and a solid/solvent ratio of 1:15 for 30 min or longer (as given in Figure 2).

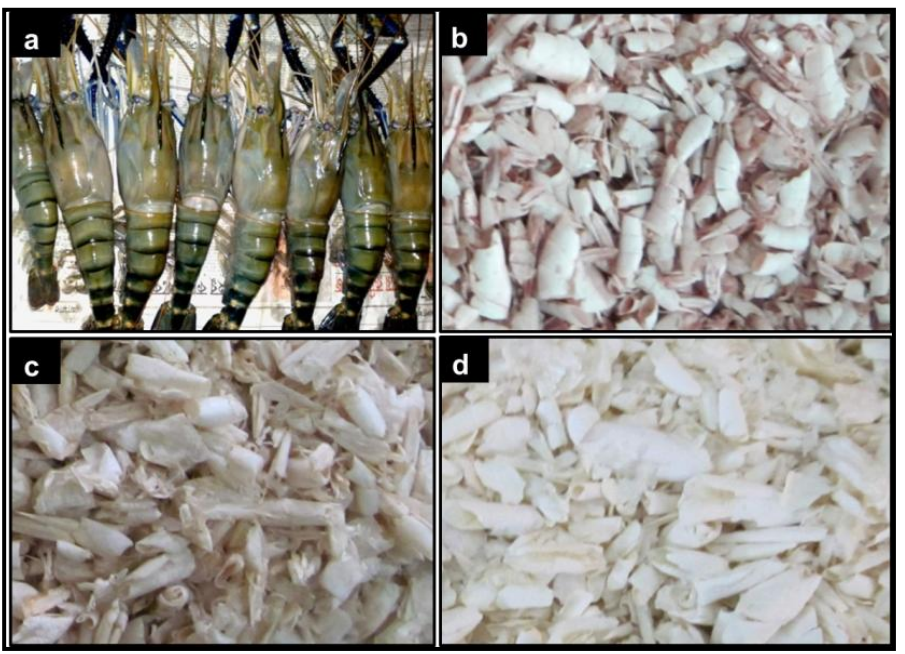
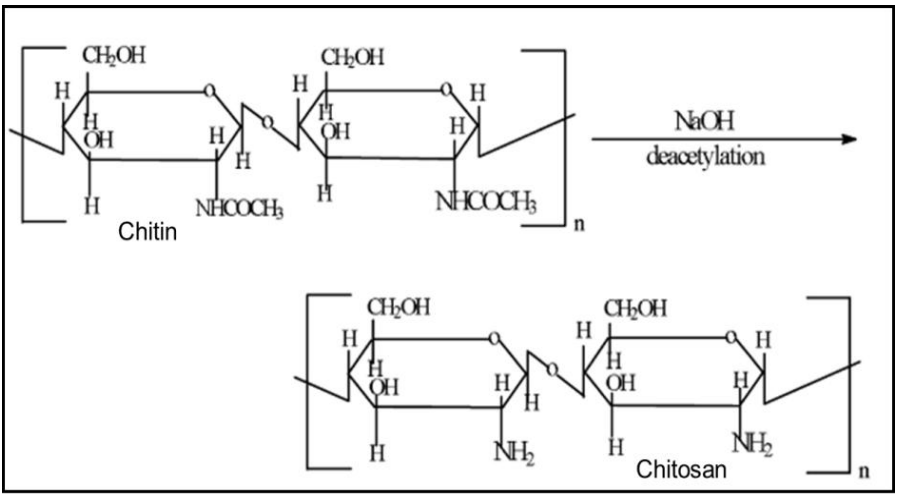


Figure 1. Fresh prawn (a), processed chitin (b), decolorized chitin (c) and chitosan (d).



Scheme 2. Deacetylation of chitin to chitosan.

The degree of deacetylation (DD) of chitosan depends on conditions in the four operation stages. Figure 3 showed common mechanism of deacetylation of chitin to chitosan.

A lot of works have been done on processing conditions of chitosan extraction. According to method of Hackman [42], the shells are digested for 5 h with 2 L of 2 N HCl at room temperature and then are extracted for 2 days with 500 ml of 2N hydrochloric acid 0°C. The collected material is washed extracted for 12 h with 500 ml of 1N sodium hydroxide at 100° which is repeated for four times to get chitosan with 17% yield. Whistler & BeMiller [43] have found 20% yield by soaking the ground shell for three days in 10% sodium hydroxide solution followed by washing with 95% ethanol and treatment with 37% hydrochloric acid solution at 20°C for 4 h.

Horowitz, Roseman & Blumenthal [44] treated decalcified shell for 18 h with concentrated formic acid (90%) and then for 2.5 hr with 10% sodium hydroxide solution on a steam bath which gave better yield of 60-70%.

Foster & Hackman [45] formulated a milder method for the isolation of chitin, based on the use of EDTA at pH 3.0, extraction with ethanol for pigment removal and with ether for lipoid removal followed by deproteinization and deacetylation processes respectively.

4. HIGH ENERGY IRRADIATION ON TO BIODEGRADABLE POLYMERS

The irradiation of polymeric materials has received much attention because it can produce diverse changes in chemical structure and physical properties, the most important of which include cross linking, degradation, oxidation, change in crystallinity, formation of unsaturation and change in melting behavior. Thus, studying the chemical and structural changes of polymers is important in practice to achieve optimal conditions for the modification of polymers [46]. Radiation processing is based on the use of high energy ionizing radiation especially gamma rays (sometimes lower ultraviolet) [47] to induce chemical and biological changes in biodegradable polymeric systems. Since high energy electromagnetic and particle radiation exhibit properties of controlled penetration and intensities, which are especially suitable for synthesis and modification of polymeric biomaterials without the need of usually toxic additives, the interest to use radiation techniques in biotechnology and biomedicine is growing rapidly. The mechanism of cross-linking by radiation is either free-radical or ionic. Cross-linking by interaction with high-energy radiation sources occurs through both mechanisms [48]. Irradiation is a very prominent and convenient method for curing initiation and becoming a popular method for producing free radicals suitable for initiating polymerization within a macromolecular substrate [49]. The basic purpose of irradiation is to ionize and excite, and both excited molecules and ions yield free radicals which initial photo-curing reaction. By this technique, many new polymers can be cured to the substrate and it is possible to characterize the grafted polymer with respect to size, number and position of the polymer chains. It was found that the radiation methods for the preparation of photo-curing are often easier to handle than most convenient chemical techniques.

5. GAMMA IRRADIATION ON CHITIN

The properties such as degree of deacetylation, solubility, crystalinity etc. properties of chitin can be greatly influenced by the application of high energy gamma irradiation on chitin. It was found that the DD of chitosan samples prepared from irradiated chitin samples was higher than the DD of chitosan samples prepared from non-irradiated chitin under similar deacetylation

reaction conditions. Even low dose irradiation has been found to make chitin more susceptible to N-deacetylation. The reason for radiation-induced enhancement of N-deacetylation was found to be mostly due to reduction in the molecular weight of irradiated chitin. Milder N-deacetylation conditions were achieved by low dose gamma irradiation of chitin [23]. Rahman et al. [22, 50] investigated extensively about the variation of DD with irradiation doses and alkali concentration during deacetylation of chitin. It was observed that that DD increases with increasing irradiation doses on chitin as shown in Table 1 and 2. It was observed that γ-irradiated chitin showed significantly higher DD than that of nonirradiated chitin sample. The increase in DD by γ-irradiation results the decrease of molecular weight and crystallinity of chitin thus promotes N-deacetylation of chitin into chitosan.

Table 1. Determination of degree of deacetylation (%DD) of chitosans produced by varying heating time and irradiation doses

Sample	γ-irradiation doses on prawn shell (kGy)	Heating time of deacetylation (h)	DD (%)
Chitosan	0	2	72.49
	0	3	73.80
	0	4	74.70
	2	2	73.17
	2	3	73.84
	2	4	75.54
	5	2	73.71
	5	3	74.11
	5	4	76.27
	20	2	73.95
	20	3	74.35
	20	4	79.69
	50	2	75.58
	50	3	79.73
	50	4	84.56

Table 2. Effect of irradiation on DD of chitin as treated with 20% NaOH solution for 2 h

γ-irradiation doses on chitin [kGy]	DD [%]
0	67.70
2	69.5
5	71.27
20	73.69
50	81.49

Rahman et al [22] also described that alkali concentrations and heating time of chitin with alkali during deacetylation process also affects DD of chitosan. For example, 10 kGy irradiated chitin deacetylated 2 hrs with 20% NaOH yielded DD of 78.5%. Furthermore 50 kGy irradiated chitin, deacetylated with 20% NaOH and 2hrs heating resulted 81.5% DD. The details results on the effect of heating time and alkali concentration on DD of chitosan for 50 kGy irradiated chitin are given in Table 3. The results demonstrated that the DD increases with heating time in alkaline solution which eventually increases hydrolysis of acetamide to amine thus reduces the percentages of N-acetylglucosamine units in chitosan. It was observed that irradiated chitin results significantly higher DD at considerably lower alkali concentration than nonirradiated chitin.

Table 3. Effect of heating time and alkali concentration on DD of 50 kGy irradiated chitin

NaOH [%]	Heating time [hrs]	DD [%]	NaOH [%]	Heating time [hrs]	DD [%]	NaOH [%]	Heating time [hrs]	DD [%]
10	4	72.3	15	4	74.2	20	4	81.5
10	3	60.9	15	3	66.1	20	3	73.7
10	2	56.7	15	2	64.7	20	2	67.3

The results revealed that the DD of chitosan increases gradually with increasing radiation doses on prawn shell. At higher radiation doses the DD of chitosan was found significantly higher than non-irradiated sample. Furthermore, the heating time in alkaline solutions during deacetylation also influenced the DD. The increase in DD by irradiation was interpreted as a result of reduction in molecular weight and decrease in crystallinity of chitin which provided the possibility of N-deacetylation of chitin into chitosan at a

mild reaction conditions. The DD of chitosan obtained from 50 kGy γ-irradiated prawn shell heated for 4 hours in alkali solution was 84.56% thus indicated the superior quality of chitosan. The changes of DD due to irradiation effects can be well observed by FT-IR of nonirradiated and irradiated chitosan samples as explained by Rahmanet al [22,50] and the spectra of irradiated chitin, nonirradiated and γ-irradiated chitosan are shown in Figure 2. The decrease of the intensities of >C=O after irradiation which occurs due to the deacetylation of N-acetylglucosamine units and the reduction of viscosity further ascertained the explanation that irradiation causes the degradation of polysaccharides. In the spectra for irradiated chitosan (b-e) there were no sharp absorption at 3500 cm^{-1}, which confirms that the hydroxyl groups in positions C2 and C6 of the chitosans are involved in intra- and intermolecular hydrogen bonds. The region above 3000 cm^{-1} was centred at 3480 cm^{-1} in chitosan and the sharpness of the peak at 3480 cm^{-1} exhibited a progressive weakening of the bands at 3265 cm^{-1} and 3100 cm^{-1} during *N*-deacetylation, indicated a higher DD with increased irradiation doses.

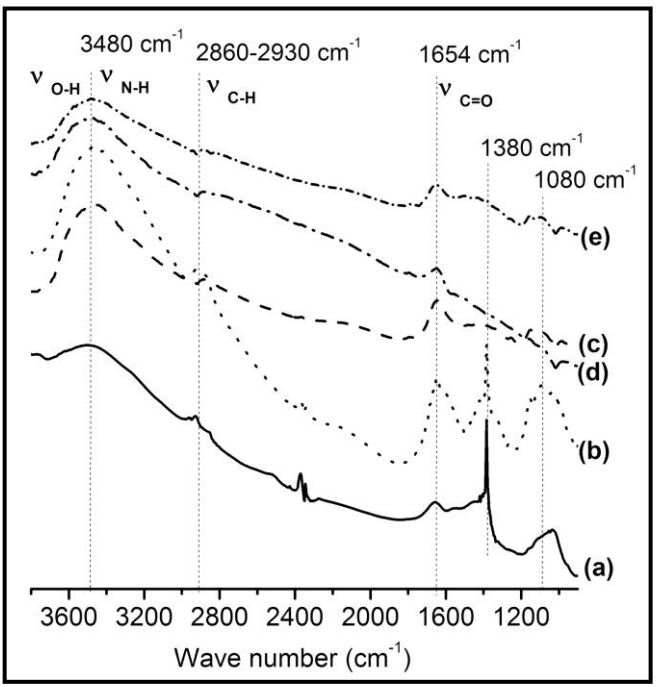


Figure 2. FT-IR spectra of non-irradiated and irradiated chitosan samples; (a) chitin, (b) non irradiated chitosan, (c) 5 kGy, (d) 20 kGy and (e) 50 kGy irradiated chitosan.

Tahat et al. [23] also examined the effect of gamma irradiation on the extraction of chitin and chitosan from gamma irradiated prawn shell by varying several parameters of the deproteination and demineralisation processes, such as the concentrations of alkali and acidic media, the reaction times and temperatures. It was found that the irradiation dose at 25 kGy, allows reducing the deproteination time (in a same conditions) by a factor of three, comparatively with non-irradiated samples. The best conditions for chitin extraction were found to be as follows: for the deproteination, irradiation at the dose of 25 kGy, NaOH concentration 1N, reaction temperature 85°C, reaction time 1h. For the demineralisation, irradiation dose 25 kGy, HCl concentration 1N, at room temperature for 3h. The best conditions for the deacetylation were as follows: NaOH concentration 60%, treatment temperature 100°C, reaction time 120 min. These conditions allow reaching a DD of almost 93%.

6. Application of Ultraviolet Radiation on Chitosan

A lot of investigations have been done to reveal the influence of UV radiation on chitosan, its derivatives, blend products, chitosan films. UV radiation can initiate free radical reactions to enhance grafting properties of chitosan with several monomers. Sionkowska et al. [51,52] investigated the influence of UV radiation on surface properties of chitosan film. He found surface free energy was altered and chemical and structural changes occurred in chitosan film during UV irradiation. It was observed that the characteristics of chitosan-collagen blend had been significantly altered by solar radiation. They also compared the effect of solar UV radiation on collagen-chitosan blend to artificial UV radiation. Another report of Sionkowska [53] has shown that the mechanical properties like breaking strength, percentage elongation and Young's modulus of the chitosan/keratin films were greatly affected by UV irradiation, but the level of the changes of these properties was smaller in the blend than in pure chitosan and strongly dependent on the time of irradiation and composition of the samples and the changes point to greater susceptibility of chitosan to photooxidation in the presence of keratin. A research of Praxedes et al. [54] showed that the immobilization of dansyl groups in the chitosan backbone leads to a pronounced enhancement of the UV sensitivity of polymeric films.

Several researchers have investigated the effect of UV radiation on different blends of chitosan. Ferdous et al. [55] have studied on mechanical properties like tensile strength (TS), elongation at break (Eb) of photocured Films of chitosan and chitosan/PVA and PEO/PVA blend with acrylic monomers and found the bioblend film PVA/chitosan performed better physico-mechanical properties rather than PVA/PEO. Khan et al. [56] have prepared by soaking chitosan film in five formulations developing with 2-ethyl-2-hydroxy methyl-1,3-propandiol trimethacrylate (EHMPTMA), a trifunctional monomer and 2-ethylhexyl acrylate (EHA), a monofunctional monomer and in the presence of photoinitiator Darocur-1664 (2%) and irradiated under UV-radiation at different radiation intensities. Then they tested different characteristics test likeTS, Eb, polymer loading (PL), water absorbency, gel content etc and found the formulation, containing 25% EHMPTMA and 73% EHA showed the best performance at 10th UV passes of UV radiation for 4min soaking time. *Bajer and Kaczmarek [57] studied on UV irradiated films of chitosan-starch blends and found that the chitosan-starch blends were more susceptible to UV irradiation comparing to pure starch specimen.* Tsuda et al. [58] found that UV light on photocrosslinkable chitosan resulted in an insoluble and flexible hydrogel within 30 s. The study also evaluated the ability of the photocrosslinkable chitosan hydrogel to inhibit bone formation in the rat skull and fibula defects for 8 weeks. Ishihara et al. [59] applied the same photocrosslinkable chitosan hydrogel on a cut mouse tail and found that it could completely stop bleeding. It also could firmly adhere two pieces of sliced skins of mouse to each other and significantly induce wound contraction and accelerated wound closure and healing.

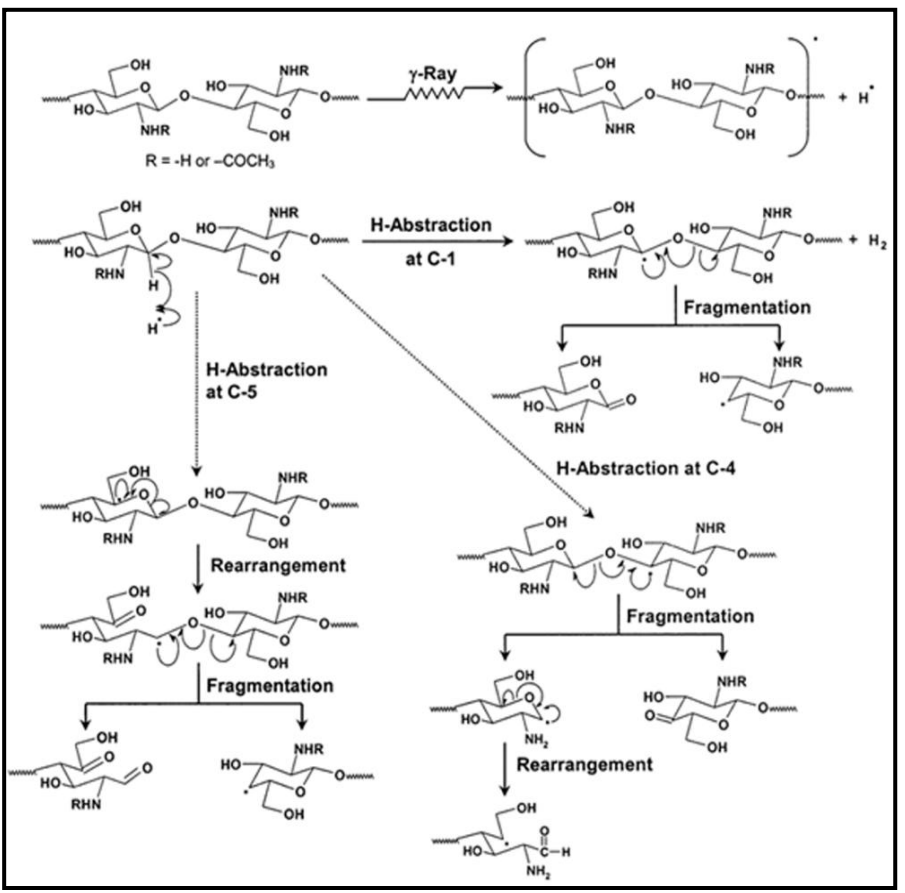
7. GAMMA IRRADIATION OF CHITOSAN

Ionizing radiation (gamma) can produce electronic excitation as well as ions and may also produce ions and free radicals in excited states. Thus, it is important that possible chemical effects due to electronically excited groups as well as to ionized groups be borne in mind. The most important chemical changes that occur during the gamma irradiation of chitosan are those of cross linking and degradation.

From the viewpoint of mechanical properties as polymer materials, cross linking of polymers leads to increasing hardness, tensile strength, elastic modulus and softening temperature, and to decreasing elongation to failure and solubility to solvents.

On the other hand, degradation of chitosan due to main chain scission leads to the opposite effects on the mechanical properties, and eventually produces soft, gummy or tar-like materials. When chitosan are irradiated, both cross linking and degradation often occur simultaneously.

Rahman et al. [22] described that γ-irradiation also brought significant changes in physico-chemical; thermal and morphological properties of chitosan and the materials afterwards can be suitable for many potential fields in biomedical and agriculture etc.



Scheme 3. Effect of gamma radiation on chitosan.

8. Effect Gamma Irradiation on Properties of Chitosan

Several studies confirmed the significant changes in chitosan properties due gamma irradiation. γ-irradiation brings a great change in physicochemical, thermo-mechanical and morphological properties of chitosan which provides the great potential for many applications of chitosan.

8.1. Molecular Weight and Viscosity

Rahman et al. [22,50] investigated the effect of gamma radiation on chitosan within the range of 2-100 kGy. The investigation showed that irradiation causes significant decrease in molecular weight of the chitosan (as shown in Figure 2) e.g. molecular weight of chitosan of 187,128.43 Da decreased to 64,972.69 Da after 100 kGy irradiation. Figure showed the decrease in molecular weight of chitosan with increasing radiation doses. The degradation of molecules occurs rapidly within the range of 2-20kGy but at higher radiation dose the change becomes insignificant. Lim et al. [21] investigated the same effect up to 25 kGy and found the same result. They suggested that the decrease of molecular weight occurred due to the chain scission of chitosan molecules at glycosidic linkage under high energy ionizing γ-radiation. All these research also revealed that the intrinsic viscosity of chitosan decreases with increasing radiation dose.

Lam and Diep [60] reported that the average molecular weight of a 90% deacetylated chitosan is reduced from 560,000 Da to 139000, 112000 and 72500 Da when irradiated at 40, 75 and 100 kGy in air medium, respectively while Gryczka et al. [61] demonstrated that a dose of 300 kGy in air medium reduces the average molecular weight of chitosan from 690000 to 20900 Da. Vilcáez and Watanabe [62] found much lower molecular weight chitosan by applying 500 kGy irradiation. Wasikiewicz et al. [63] compared three degradation methods of degradation of chitosan and alginate like ultrasonic, ultraviolet and gamma irradiation. After monitoring the molecular weight by GPC measurement, they found that from the energetic point of view the most effective method for both polymers was gamma radiation method and considering the reaction time, the ultraviolet method is the most effective. By investigating FTIR spectra, taken before and after degradation it was revealed, that degradation undergoes by the breakage of the glycosidic bonds of

polymers. Tahtat et al. [64] found the degradation of chitosan molecules by gamma radiation were faster in aqueous state than solid state. They also mentioned that chitosan with higher molecular weight were more sensitive to radiation than low molecular weight chitosan.

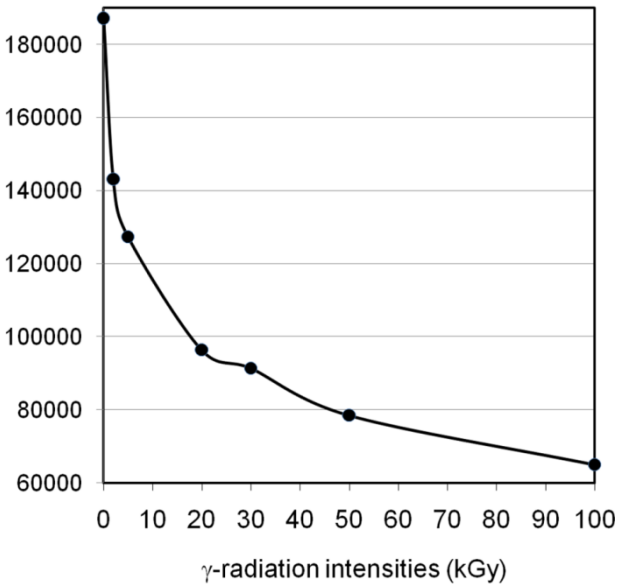


Figure 3. Variation of molecular weight of chitosan with irradiation doses [40].

Kang et al. [65] was observed that that in the degradation with a combination of gamma radiation and hydrogen peroxide, the rate of decrease in molecular weight was much higher than that in the degradation with gamma radiation or hydrogen peroxide process alone. The results suggest that the synergetic effect is operative in the degradation of chitosan with gamma radiation and hydrogen peroxide. The formation of OH radical is enhanced through the radiolysis of H_2O_2 or water. Hydroxyl radicals, as powerful oxidizing species, can attack the b-1-4 glycosidic bonds of chitosan and enhances the degradation. A study of Yoksan et al. [66] estimated the optimal conditions for γ irradiation to reduce the molecular weight of chitosan but still retain its chemical structure. Chitosan was irradiated under various conditions, i.e. flake solid state, flake dispersed in water, flake dispersed in 0.05, 0.1, 1 and 2% aqueous $K_2S_2O_8$ solution, flake dispersed in 0.5, 1 and 2% aqueous H_2O_2 solution, and chitosan acetic acid solution. Comparative studies were done using three types of chitosans with molecular weights of the order of 10^5

Da with degrees of deacetylation of 0.80, 0.85 and 0.90%. A severe degradation occurred in the first region with decreases in the molecular weight of 80% for radiation doses up to 50 kGy for flake solid state, flake dispersed in water and flake dispersed in 0.05, 0.1, and 1% aqueous $K_2S_2O_8$ solution and 20 kGy for flake dispersed in 0.5, 1 and 2% aqueous H_2O_2 solution. The degradation of chitosan by γ rays was found to be most effective for the amorphous structure.

9.3. Effects on WBC and FBC

According to Kim et al. [67] and Youn et al. [68] the lower the molecular weight the higher the WBC; the higher the DD the higher the WBC and lower the crystallinity. All these facts determined the increase in WBC with increasing radiation dose. Rahman et. al [22] have found WBC 1210.28% for 100 kGy irradiated chitosan while for non irradiated chitosan it was 627.32%. They also suggested that the increased surface area due to the decomposition of chitosan increased the area for binding with the -OH group, $-NH_2$ group and the end groups. The increased DD due to radiation provided more - NH_2 group to bind water and the decrease in crystallinity increases the penetration of the water molecules more easily. The fat binding capacity of chitosan depends on the decree of deacetylation of chitosan mainly. The fat molecules which are fatty acid glycerides bind with the amine group of the chitosan. Generally a chitosan molecule can absorb 8 to 10 times of its weight of fat molecules. It was also described in our recent studies [22] that WBC as well as the FBC of chitosan also increases with the increasing irradiation doses as shown in Figure 4. The FBC of normal chitosan was 574.03% where after radiation of 2kGy, 5kGy, 20kGy, 30kGy, 50kGy and 100kGy the FBC were found as 962.7, 1153.2, 1396.7, 1461.3, 1535.4 and1573.4% respectively. The decrease in molecular weight and the decrease in crystallinity with radiation also affected the FBC of chitosan.

9.4. Effects on Thermal Properties

Differential scanning calorimetry (DSC) and thermo gravimetric analysis (TGA) are well established method to evaluate thermal stabilities of polysaccharides and other polymeric materials. DSC and TGA thermogram of irradiated chitosan in comparison with nonirradiated samples are graphically

presented in Figure 5 and 6. The results revealed that the exothermic peaks (decomposition temperature) within the region 290°C-310°C slightly moved to the left with increasing radiation dose i.e. the decomposition temperature of chitosan decreased with the increasing radiation dose (Figure 5). The same result was observed in case of glass transition points (endothermic peak in the middle) within the region 230°C-260°C and in the case of moisture removal (broad endothermic peak) within the region 70˚C-90°C. The changes were more visible in case of glass transition temperature and decomposition temperature rather than in case of moisture loss temperature. The degradation of chitosan molecules with the gamma radiation was responsible for that. The chitosan with lower molecular weight degraded at lower temperature than higher molecular weight chitosan. Besides as the chitosan molecule decomposed with increasing radiation dose, the degree crystallinity of chitosan also decreased which helped to decrease the glass transition temperature of the chitosan. Radiation had negligible effect on moisture loss temperature as it did not depend on molecular weight largely. Rahman et al [50] have also found similar trend for 2-50 kGy irradiated chitosan. Fajardo et al. [69] suggested that there was a chance of the evolution of NH_3, CO_2, NO_2,NO, N_2 gases from solid chitosan molecule because of bond breaking at high intensity of γ-radiation.

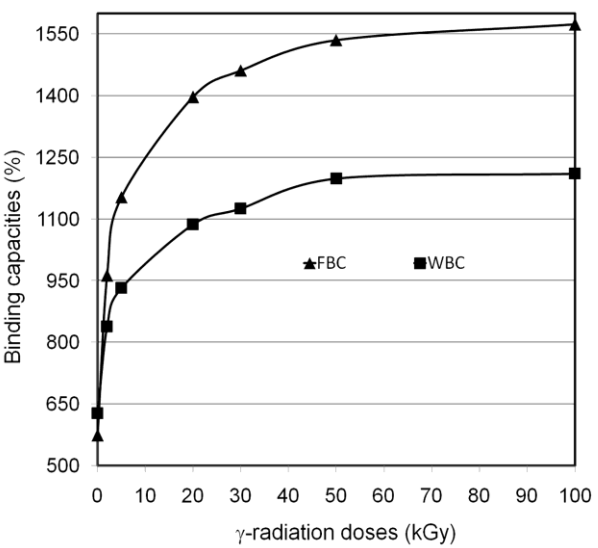


Figure 4. Effect of gamma radiation on WBC and FBC.

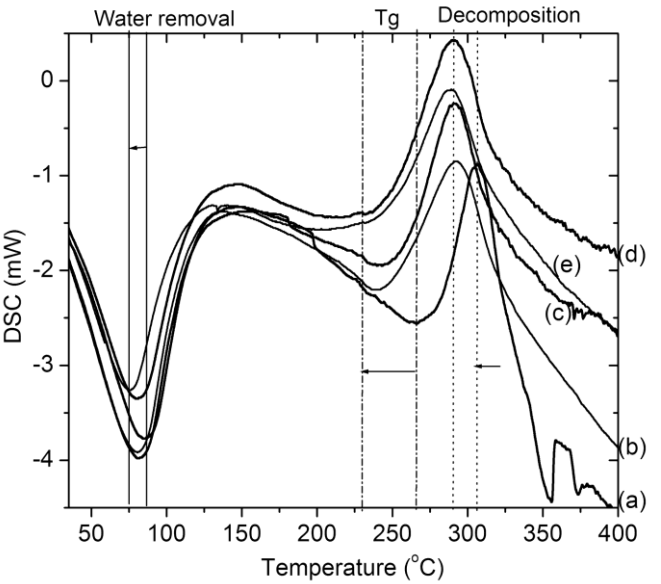


Figure 5. DSC thermogram of non irradiated and irradiated chitosan; (a) non irradiated chitosan, (b)20 kGy, (c) 30 kGy, (d) 50 kGy and (d) represents thermogram of 100 kGy γ-radiation doses [40].

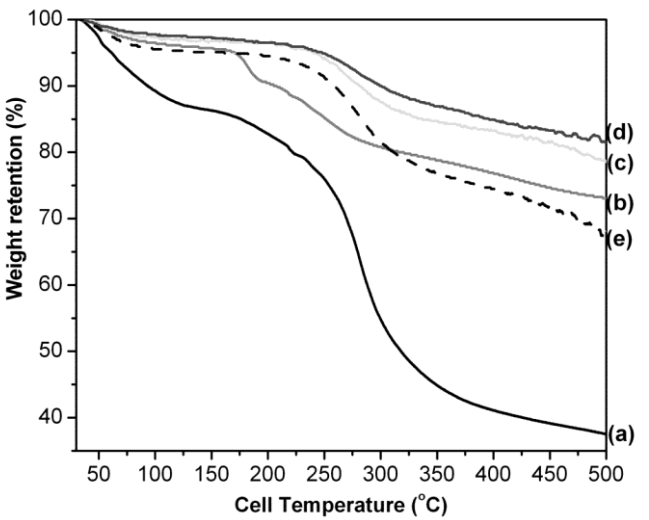


Figure 6. TGA thermograms of (a) non irradiated, (b) 10 kGy, (c) 30 kGy, (d) 50 kGy, (e) 50 kGy and (f) for 100 kGy irradiated chitosan.

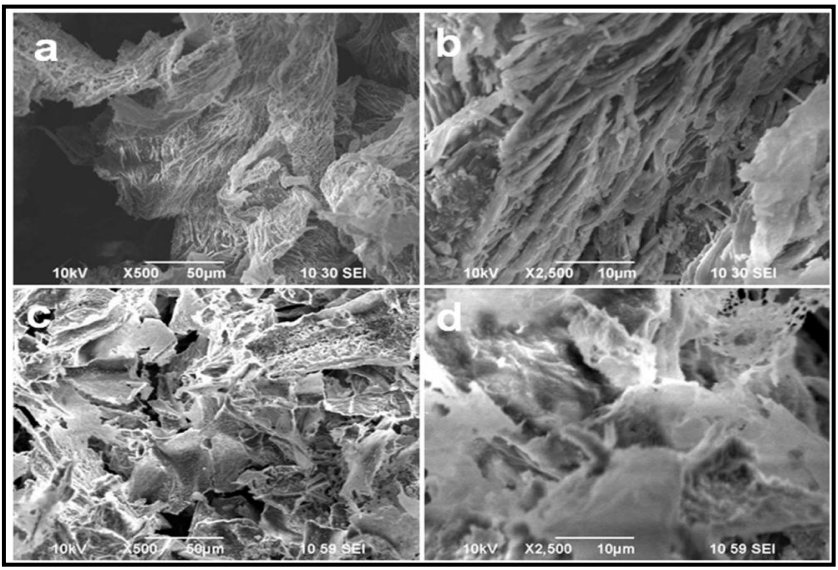


Figure 7. Scanning electron micrographs of nonirradiated chitosan (a and b) and 100 kGy irradiated chitosan (c and d).

Thermogravimetric analysis of non irradiated chitosan, and irradiated chitosan from 10-100kGy also confirmed the change in degradation temperature of chitosan with the gamma radiation. The degradation of irradiated chitosan started at least 50˚C earlier than that of the normal chitosan although it was slower in case of irradiated chitosan (Figure 6). There was slight transition in degradation temperature of 100 kGy irradiated chitosan from 30 kGy irradiated chitosan.

8.4. Effects on Surface Morphology

Surface morphologies of non-irradiated and 100kGy irradiated chitosan were studied by Rahman et al [22] by scanning electron microscopic analysis. It was demonstrated that the non-irradiated chitosan particles had uniform network and were agglomerated. After irradiation by 100 kGy the chitosan chain were ruptured and irregular arrangements of the molecules were observed. The SEM of 20 kGy and 50 kGy irradiated chitosan also confirmed that chitosan molecules were degraded with application of gamma irradiation as was given in Figure 7.

8.5. Effect on Antimicrobial Properties

Chitosan shows two types of antimicrobial activities. It inhibits the growth of microorganisms. Addition to this, gamma irradiated chitosan becomes microorganism free chitosan due to ionizing radiation effect. Chowdhury et al. [70] have found that in the anti-bacterial activity of irradiated and un-irradiated chitosan, it is effective for the inhibition even at the concentration of 0.025 %. 0.1 % chitosan was quite efficient in killing five different strains of bacteria tested with high microbial load. Rahman et al. [22,50] have also investigated antimicrobial test of chitosan for *Bacillus sp* and *E. coli* and found zero count after 96 h (as shown in Figure 8). Sekiguchiet et al. [71] have suggested that both molecular weight and degree of deacetylation affected the antimicrobial activity of chitosan independently, though the influence of the Mw on the antimicrobial activity is greater than the influence of the DA. Jung et al. [72] found the lower molecular weight chitosan showed better result against microbial growth while chitosan antimicrobial effectiveness is improved as the degree of acetylation is lower. Anti-bacterial activity of irradiated chitosan has aslo been tested against *Escherichia coli* B/r by Matsuhashi and Kume [73] and found that chitosan at 100 kGy irradiated in the dry state is effective in increasing the activity, and inhibited the growth of *E. coli* completely at a concentration of 3 mg/ml. They also induced Anti-fungal activity but were lower than the anti-bacterial activity. Lam and Diep [60] studied on radiation treatment of chitosan for enhancement of antifungal activity tested on fruit- spoiling strains. They have indicated that degree of deacetylation of chitosan clearly affects its antifungal activity, the higher the deacetylation of chitosan, stronger antifungal activity can be observed that also demonstrated that chitosan irradiated at doses higher than 20 kGy significantly increased clearly the antifungal activity.

9. RADIATION INITIATED GRAFT COPOLYMERIZATION OF CHITOSAN

Both high- and low-energy radiation may be used forgraft copolymerization of vinyl monomers onto polysaccharides. Employing high-energy radiation (e.g., β, γ, X-ray) is an efficient basic method for initiating radical graft polymerization onto polysaccharides. The initiation method with the highest graft efficiency seems to be pre-irradiated of the polysaccharides

with gamma radiation followed by the activated polysaccharide with vinyl monomers under suitable reaction conditions [74, 75]. Grafting efficiency means in this connection not only a high number of grafted branches with high molecular weights, it means in particular a low level of homopolymer formed. Although the radiation-based grafting is cleaner and more efficient in this regard than chemical initiation methods, they are harder to handle under technical conditions [74]. Irradiation of gamma-ray on powdery chitin initiates the graft polymerization of styrene, as in the case of cellulose, but the grafting percentage is low (i.e., 64%). Styrene was also graft copolymerized onto chitosan powders or films. Water was recognized to be essential for the both grafting reactions. The chitosan-*graft*-polystyrene adsorbed bromine better than chitosan, and the copolymer films showed less swelling and higher elongation in water than what the chitosan films did [76,77]. Pengfei et al. [78] recently reported the gamma-radiation induced graft copolymerization of styrene onto chitin and chitosan powder. The polydispersity index of the grafted chains was measured to be basically between 1 and 2. Singh and Ray graft copolymerized 2-hydroxyethylmethacrylate (HEMA) onto chitosan films using ^{60}Co gamma radiation to improve their blood compatibility [79].They found that the level of grafting could be controlled by the grafting conditions, namely solvent composition, monomer concentration, dose rate, and total dose. They achieved a maximum graft yield of 108% under the conditions of solvent water-methanol volume ratio 1:1, HEMA concentration 20 vol%, dose rate 90 rad/s and total dose 0.216 Mrad. The swelling of this PHEMA-grafted film in phosphate buffer (pH7.4, 0.1 M) at 37°C was 58% compared to that of the original chitosan film, i.e. 110%. Chitosan films have also been subjected to gamma radiation-induced graft copolymerization of the vinyl monomer *N*,*N*-dimethylaminoethyl methacrylate [80]. The reaction variables affecting on the grafting percentage have been studied. The grafting of 70% is being achieved under the conditions of solvent water methanol volume ratio 1:1, the monomer concentration 15 vol%, dose rate 90 rad/s and total dose 0.216 Mrad, and irradiation time 80 min. The garfted chitosan was fully characterized by swelling and tensile measurements, and FTIR, DSC, TGA and XRD methods. The degree of swelling, crystallinity, and tensile strength were decreased by 51, 43, and 37%, respectively, at a graft level 54%, whereas the modified films showed improved thermal stability [80]. Low energy photons may also initiate the polymerization of a vinyl or acrylic monomer if the irradiation is carried out in the presence of an activator (photosensitizer). Such a sensitizer must become active on exposure to the particular wavelength range of the incident radiation [81].

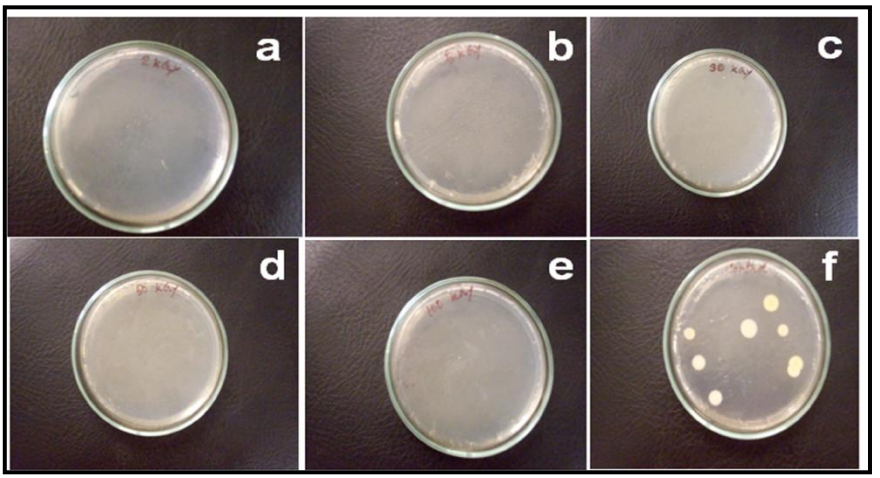


Figure 8. Microbial test plates after 24 hours of incubation for (a) 2 kGy, (b) 5 kGy, (c) 30 kGy, (d) 50 kGy, (e) 100 kGy irradiated chitosan and (f) non irradiated chitosan.

10. Diverse Application of Gamma Irradiated Chitosan

Rahman et al. [50] have potentially applied gamma irradiated chitosan solution for plant growth promotion that showed extremely enhanced growth for *Malabar spinach*. The chitosan solution was sprayed on to the specimen plants and neighboring soil where germinations were taken place. Through the investigation of plant height, number of leaves, leaf areas, dry and wet weight of the plants and roots they concluded that 30 kGy irradiated chitosan yielded 60% higher growth of the *Malabar spinach* than that obtained from nonirradiated chitosan (as given in Figure 9).

In addition to plant growth stimulator supplementation with optimum concentrations of 200 kGy irradiated chitosan resulted in a significant increase in the fresh biomass (68.1% for chrysanthemum, 48.5% for lisianthus, 53.6% for limonium and 26.4% for strawberry), shoot height (19.4% for chrysanthemum, 16.5% for lisianthus, 33.9% for limonium and 25.9% for strawberry) and root length (40.6% for chrysanthemum, 66.9% for lisianthus, 23.4% for limonium and 22.6% for strawberry) [82]. It also enhanced the activity of chitosanase in treated plants and also improved the survival ratio and growth of the transferred plantlets acclimatized for 10-30 days under greenhouse conditions.

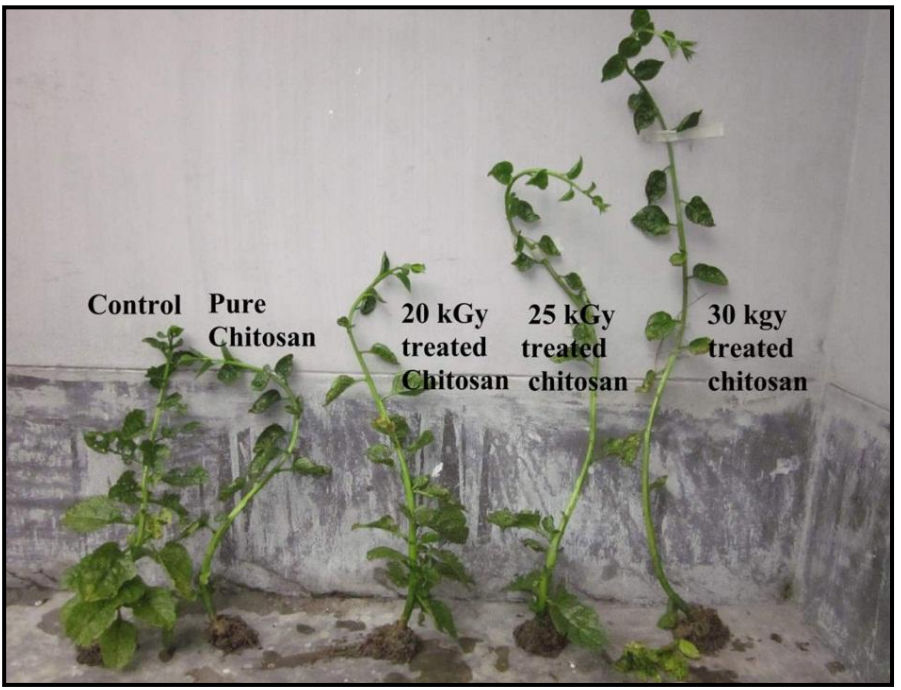


Figure 9. *Malabar spinach* plants of 41 days age obtained by applying irradiated and nonirradiated chitosan.

A group of scientist showed a remarkable effect of a special fraction F2 of irradiated chitosan on the growth of barley and soybean, and significant increase in activity of phytoalexin enzymes, namely phenylalanine ammonia lyase (87%) and chitinase (186%). It also increased 15.8% seed yield of soybean after three month cultivation. The results suggested that the irradiated chitosan fraction F2 with Mw in range of 1-3 kDa was a trigger for plant growth activities [83]. Irradiated chitosan also used in preservation of different kinds of foods like papaya [84], different types of fruits [85], processed lamb meat [86] etc. Raw chitosan under acid conditions (pH 6.3) exerted the strongest inhibition against a mixed culture of denitrifying bacteria followed by the 100 kGy and 500 kGy irradiated chitosans, respectively. As the molecular weight of chitosan decreases with the degree of γ-irradiation, the inhibitory properties of chitosan due to its high molecular weight were more relevant than the inhibitory properties gained due to the modification of the surface charge and/or chemical structure by γ-irradiation. Gamma-irradiated chitosan microparticles, exhibited a slightly higher drug release rate and low

swelling capacity than the nonirradiated microparticles [87]. The study also reported that there were no notable changes in encapsulation efficiency of irradiated microparticles. Chitosan and hydroxyethyl methacrylate (HEMA) matrix have been prepared by γ-irradiation which could be used as wound dressing material and as a controlled drug release system [88]. Antibiotic release experiment Results pointed out a fast amoxicillin release with similar release profile in all studied membranes.

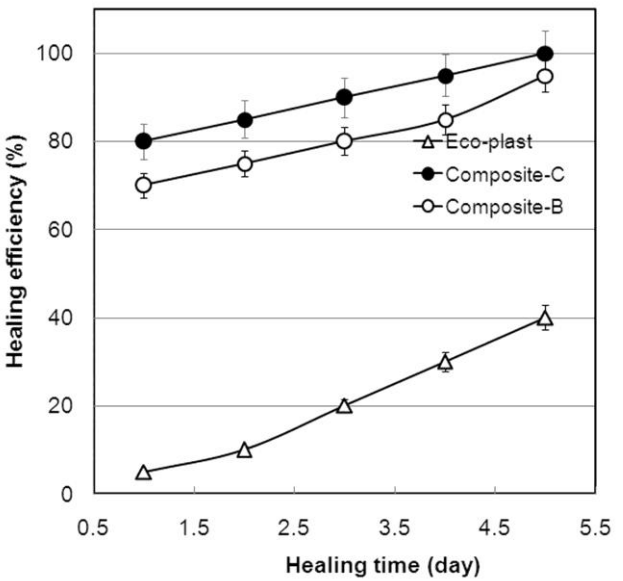


Figure 10. Wound healing efficiencies of Whistar rat by using chitosan composites and commercial scaffold (Eco-plast).

Jin et al. [89, 90] prepared scaffolds biocomposite from chitosan and gelatin for utilizing as a promising matrix for tissue engineering. Jin et al. studied different properties of the scaffold and suggested to use as a promising matrix for tissue engineering. The chitosan-gelatin blends with various compositions were potentially tested on Wistar rat model and found suitable as candidate materials for biomedical applications that also demonstrated good compatibility between these two biodegradable polymers. The composite films showed improved tensile properties, highly porous structure, antimicrobial activities, low water dissolution, low water uptake and high buffer uptake compared to pure chitosan or gelatin films. The chitosan scaffold with gelatin at a ratio of 3:10 showed 100% healing in the Wistar rat; whereas the

commercial Eco-plast showed only a little above 40% healing of the dissected rat wound (as given in Figure 10).

Porous scaffolds for skin tissue engineering were fabricated by freeze-drying the mixture of collagen and chitosan solutions by Ma et al. [17]. They found that a steady increase of the biostability of the collagen/chitosan scaffold was achieved when GA concentration was lower than 0.1%, then was less influenced at a still higher GA concentration up to 0.25%. In vitro culture of human dermal fibroblasts and the in vivo studies revealed that the scaffold could sufficiently support and accelerate the fibroblasts infiltration from the surrounding tissue.

CONCLUSION

Chitosan has been shown as a very promising biomaterial with numerous fields of application. Irradiation processes enhance the usability of chitosan in a large extend. The increase in solubility of limited soluble chitosan by gamma irradiation makes it for easier application. Most of the properties of chitosan can be altered through irradiation and optimum conditions may lead to facile uses of chitosan. In the presented review we have focused on how the properties of chitin and chitosan can be altered by the application of high energy gamma irradiation. It was revealed that by simply applying high energy irradiation and without using any chemicals the properties of chitosan can be greatly altered which might give scientist more interest towards such polysaccharides for their new end uses from biomedical to agriculture or in tissue implant. Due to improvement in solubility, antifungal and antibacterial activities by gamma irradiation, chitosan is now considered as pioneer plant growth promoter in agricultural and a suitable material for food preservations. The increase in fat binding capacity may facilitate the use of irradiated chitosan as fat blocker/fat reducer in obese human body. However an intensive investigation should be done on residual effect of gamma radiation in irradiated chitosan and other related products.

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Chapter 3

LIPID SYNTHESIS AND TRANSPORT IN SHRIMPS

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ABSTRACT

In aquatic invertebrates, lipids represent an important source of stored energy, as well as structural components of cellular membranes and other lipoprotein complexes. In the freshwater shrimp *Macrobrachium borellii*, we demonstrated that the hepatopancreas, also known as midgut gland, has a high capacity for triacylglycerol biosynthesis, storage and breakdown. When radioactive palmitic acid was injected *in vivo*, most of the label was transported to the hepatopancreas, where triacylglycerol were actively synthesized. The enzymatic activity that initiates glycerolipid synthesis, glycerol-3-phosphate acyltransferase, is located in the mitochondria. In contrast, triacylglycerol synthesis in hepatopancreas microsomal fraction follows the monoacylglycerol pathway. Even though triacylglycerol count for the major lipid class (up to 80% of the total lipids), phosphatidylcholine is exported from the hepatopancreas to other tissues and transported in hemolymph as a high-

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density lipoprotein (HDL), suggesting that triacylglycerol are stored in the hepatopancreas for energy supply or lipid remodeling. *M. borellii*'s HDL lipid moiety is composed mainly of phosphatidylcholine with minor quantities of cholesterol and triacylglycerol. Lipid transference between hepatopancreas and HDL was studied *in vitro*, confirming that HDL releases free fatty acids to the hepatopancreas, whereas phosphatidylcholine and other phospholipids are liberated from the hepatopancreas to HDL.

Proteins, lipids and carbohydrates present in the vitellus of eggs are important energy and building block sources for the embryo development in ovipara. In aquatic invertebrates, these compounds are usually associated forming lipoproteins called lipovitellins (LV) that function as nutrient sources for the development of the embryo and also satisfy the metabolic larvae needs from their birth to the moment they start feeding on external sources.

In *M. borellii* and other crustaceans, LVs are high-density lipoproteins with phosphatidylcholine as the major lipid and minor quantities of other lipids like triacylglycerol and phosphatidylethanolamine. LVs are originated from a plasma lipoprotein restricted to ovogenic females: vtellogenin (VG). M. *borellii*'s VG lipids are similar quantities of phosphatidylcholine, sphingomyelin, triacylglycerol and phosphatidylethanolamine. During vitellogenesis, VG is endocyted into the ovary, where it is processed and changed into LV. Compared to VG, LV has twice as much phosphatidylcholine as the other lipid classes and less sphingomyelin, suggesting that the lipoprotein processing in the ovary provides LV with different lipid domains specific for its biological function. Due to the fact that some of LV apolipoproteins have been detected inside the developing embryo, it was concluded that LV was consumed. LV transfers to the embryo mainly the lipids loaded inside the ovary (mostly phosphatidylcholine), together with proteins to feed the embryos.

Further studies are needed to determine the lipid transference mechanisms involving the lipoproteins with lipid donor and receptor organs. However, our knowledge about lipid transport and metabolism in crustaceans has evolved in the last few years regarding both plasma and yolk lipoproteins; it sets ground for understanding the lipoprotein function in reproduction and embryo development.

Crustaceans are one of the largest and most diverse groups among invertebrates. Many species (prawns, crabs and lobsters) are related to commercial exploitation including intensive aquiculture practices. Other species, either planktonic or benthonic, play an important role in trophic chains of marine and freshwater ecosystems.

Due to their importance, studies dealing with these taxa are numerous and complex, leading to broader and more accurate interpretations of the results. In the last years, biochemical investigation has contributed to get further knowledge on the metabolic pathways of crustaceans, in general, and decapods in particular. This information is essential for developing different fields of basic research as well as biotechnological approaches. A clear example to achieve a better productive development is to know nutritional requirements and different rates of growth in many crustaceans for aquiculture purposes. Also, it is essential to know the lipid and protein composition, as well as the essential nutrient requirements of species utilized in the food industry. Additionally, knowledge on the molecular interactions in several metabolic pathways within the crustaceans, as well as between them and different organisms and/or their environment may lead to answer lots of ecological and population queries.

Biochemical-physiological studies provide fundamental information about reproductive biology, nutrition, molecular interactions with the environment, etc. Concerning molecular groups, lipids are essential components of cellular membranes. They represent an energy store much more effective than carbohydrates; their composition varies according to sex, age, nutritional and reproductive states.

LIPID METABOLISM IN CRUSTACEANS

We have been studying crustacean lipid metabolism for over two decades using the shrimp *M borellii* as a model. Firstly, the lipid and fatty acid compositions of different organs were determined, showing that seasonal distribution of lipids was closely related to the diet. Different organs (gonads, hepatopancreas, muscle and gills) were analyzed, and it was determined that the hepatopancreas was the organ where most of the energetic lipids were stored, so that during the winter season the triacylglycerol content of hepatopancreas comprised more than 80% the total lipid mass of the organ (González-Baró et al., 1988).

Then, the effect of the environmental temperature on lipids of unfed and fed shrimps was studied. During the rather long fasting period (4 weeks) energetic lipids were not consumed in the hepatopancreas at low or moderate temperatures, but lipid consumption was significantly higher under thermal stress conditions due to energy demands provoked by an increase in the water temperature (Pollero et al., 1991).

Despite the fact that adipose tissue stores triacylglycerols for energy purposes in vertebrates, in crustaceans this function is attributed to hepatopancreas, which mobilizes under thermal stress conditions (Pollero et al., 1991). This behavior was also studied by Sanches-Paz et al., (2006) using marine crustaceans during starvation.

Although it is known that hepatopancreas performs different functions as secretion of digestive enzymes and excretion of waste materials (O Connor and Gilbert, 1968, Al-Mohanna and Nott, 1986), we revealed that the hepatopancreas of *M. borellii* is also the principal organ for triacylglycerol synthesis by *in vivo* incubations with [^{14}C]palmitic acid (González-Baró et al., 1993).

The first steps in the de novo biosynthesis of glycerolipids involve the activation of fatty acids by acyl-CoA synthetases and their subsequent esterification by sn-glycerol-3-phosphate acyltransferases (GPAT). We reported that microsomes from *M borellii* hepatopancreas have a significant acyl-Coa synthetase activity (González-Baró et al. 1990). In addition we reported that the synthesis of triacylglycerols may occur in the endoplasmic reticulum of crustacean hepatopancreas (González-Baró et al., 1993), and this synthesis may be affected by toxic substances (Lavarias et al., 2007). Moreover, a mitochondrial type of acyl-Coa synthethase activity was described in this organ (Lavarias et al., 2006). The activity of GPAT in hepatopáncreas was measured by applying the methodology used in mammals where two types of GPAT activities were characterized, one in the mitocondria and the other in the microsomes. In *M. borellii* GPAT activity was mainly found in mitochondria of hepatopancreas and in a minor proportion in microsomal fractions; these studies were done *in vitro* using [^{14}C]-palmitate (Figure 1). The scarce activity present in microsomes was attributed to a possible contamination with the mitochondrial fraction because: 1) the enzyme kinetics in the microsomal fraction is similar to the one in mitochondrial fraction, 2) mitochondrial marker enzyme activities correlate the microsomal activity with the degree of mitochondrial contamination. Radioactivity distribution that was associated with the glycerolipids synthesized from [^{14}C]glycerol-3-phosphate was consistent with the products of the mammalian mitochondrial-GPAT1 reaction (Shephard and Hubscher 1969). In mitochondria the major product was found to be phosphatidic acid (56%), then triacylglycerols (32%) and diacylglycerols (11%) Figure 1. The presence of diacylglycerols and triacylglycerol suggests that *M. borellii* mitochondria contain the enzymes acyl-glycerol-3-phosphate acyltransferase, phosphatidic acid phosphohydrolase, and diacylglycerol acyltransferase. Kinetic activity of

GPAT in the mitocondrial fraction was similar to the rat and mouse isoform GPAT1. The enzyme activity was resistant to sulfhydryl reagents which altered the GPAT microsomal isoforms of mammals; also it showed a preference for saturated acyl-CoA substrates versus insaturated fatty acids (Figure 2). Polyclonal antibodies anti-GPAT1 recognized a simgle 70 kDa protein present in the mitochondrial fraction (Figure 3).

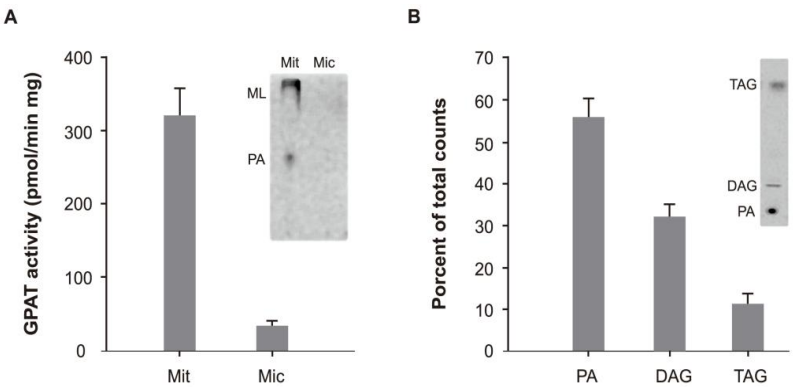

Figure 1. A GPAT specific activity in mitochondrial (Mit) and Microsomal (Mic) fractions from *M. Borellii* hepatopancreas. B Lipid classes distribution of (^{14}C)glycerol-3-phosphate esterified by mitochondria. The insets show the products of GPAT reaction resolved in TLC plates for polar (panel A) and neutral (panel B) lipids: PA phosphatidic acid; NL neutral lipids, DAG diacylglycerol; TAG triacylglycerol.

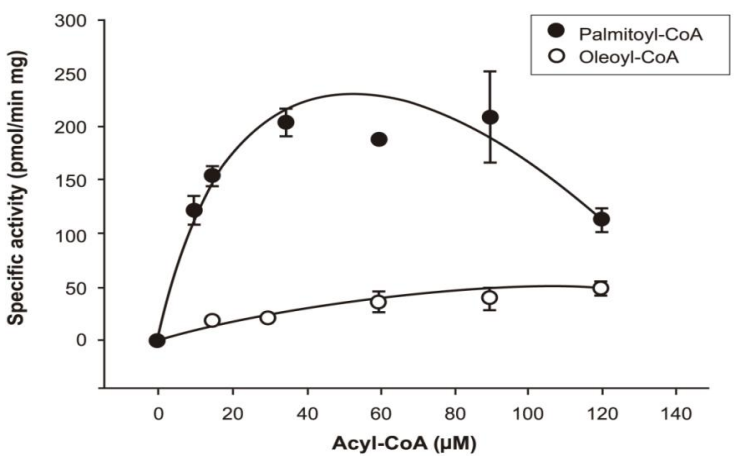

Figure 2. Acyl-CoA substrate preference of GPAT reaction in mitochondria.

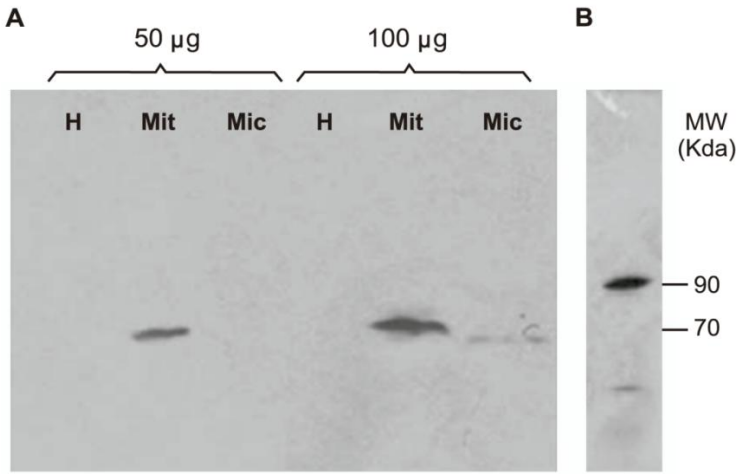

Figure 3. Mitochondria from *M. borellii* contain an immunorecutive protein against GPAT 1 antibody A: 50 ug or 100 ug of protein from total homogenate (H), mitochondria (Mit) and microsomes (Mic). B: 50 ug of rat-liver mitochondria were probed with the same antibody.

In brief, it is suggested that GPAT1 from rat-liver mitochondria and the mitochondrial GPAT from *M borellii* hepatopancreas may be homologous (Pellon-Maison et al., 2009).

Information about triacylglycerol synthesis in invertebrates is scarce. Only a single report described the incorporation of [^{14}C]-glycerol-3-phosphate into the lipids of the insect *Ceratitis capitata* and demonstrated that triacylglycerols could be synthesized in mitochondria, although a higher rate of triacylglycerols synthesis was observed in microsomes (Meier et al., 1981). Finally, we concluded that de novo synthesis of triacylglycerols is initiated in mitochondria, whereas in microsome triacylglycerol synthesis occurs through an alternate pathway, probably the monoacylglycerol pathway (Pellon-Maison et al., 2009).

LIPOPROTEINS

Lipids are associated to specific protein carriers such as blood or hemolymph proteins in order to be transported to the site of storage or metabolization. Protein components of these molecular aggregates are called apolipoproteins; they can play several functions, including hydrophobic lipid

transport in aqueous systems and signal emission to target cells. Available information on this topic is mainly related to vertebrate lipoproteins. In general they are spherical aggregates with an hydrophobic-lipid core surrounded by an envelope of polar lipids and apolipoproteins. Also, there are discoidal lipoproteins that are devoid of the hydrophobic core. Lipoproteins, their structure and functions are less known in invertebrates with the exception of insects.

HEMOLYMPHATIC LIPOPROTEINS

The presence of hemolymphatic lipoproteins has been reported in several crustacean species such as *Uca pugilator*, *Cancer magister*, *Orchestia gammarella*, *Palaemon paucidens*, etc. The presence of lipoproteins in Xiphosura *Lymulus polyphemus* (primitive group of athropods) suggests that plasma lipoproteins would have arisen early in evolution during the Cambrian Period, in a group that fossil records evidence a direct relationship with the trilobites (Lee, 1991). In some crustaceans such as *Penaeus japonicus* and *Homarus americanus*, a high amount of lipids can be transported by hemolymph (2.5 and 2.3 mg/ml, respectively).

The hemolymphatic plasma of crustaceans contains a major lipoprotein similar to vertebrate HDL, with phosphatidylcholine as predominant lipid and lower amount of cholesterol and triacylglycerols. It may also contain carotenoids. Studies of electronic microscopy revealed that in *Palinurus interruptus* these particles could be discoidal with a diameter of 122/127 Å (Lee and Puppione, 1978). Lipids are supposed to be transported by this HDL from hepatopancreas to other tissues including muscle. In hepatopancreas there is a group of cells called "R" that are able to store triacylglycerols and another group of "F" cells which would be responsible for assembing phospholipid-rich HDL (Lee, 1991).

Male and female *P. semisulcatus´* HDL has been purified by ultracentrifugation (Ton et al., 1993; Khayat et al., 1994). It contains a 110 kDa apolipoprotein and 50% lipids. HDL was also purified in *P. japonicus*; it contained 71-77% phospholipids, 18% cholesterol, 5% diacylglycerols and low amounts of triacylglycerols. As already discussed, phosphatidylcholine was the most abundant lipid, and the fatty acids 16:0, 16:1n-7. 18:0, 18:1, 18:2n-6 and 20:5n-3 were also found (Lubzens et al., 1997).

Marine crustaceans *Charybdis feriata* and *P. japonicus* HDL showed density values of 1.16 and 1.18 g/ml, respectively. They were similar to those

found in other crustaceans as well as in the decapod *Machobrachium rosenbergii* (1.13 g/ml), the crab *Eriocher japonica*, (1.16 g/ml) and in *Pacifastacus leniuscus*, (1.145 g/ml) (Hall et al., 1995). In all of them phospholipids were the predominant lipids (Komatsu et al., 1993).

Yepiz-Plascencia (2000) presented a compilation about lipoproteins of paeneids, mainly describing their protein portion.

As a part of an study dealing with the effect of xenobiotics on lipoprotein structure, we described that both plasma lipoproteins present in the freshwater shrimp *Macrobrachium borellii* were found to have high density (HDL) and a high content of phospholipids (García et al 2002), similar than previous reports (Lee, 1991, Yepiz-Plascencia et al., 2000). One of them, named HDL1, showed the predominance of phosphatidylcholine and lack of sphingomyelin. It was utilized as a model to demonstrate its role as lipid carrier between tissues (see below). This lipoprotein particle mass (290 kDa) is consistent with that one reported for a lipoprotein of similar function in the crab *Calinectes sapidus* (Lee et Puppione, 1988); it also doubles the mass of certain penaeids such as *P. semisulcatus* (Lubzen et al, 1997) and *P. vanamei* (Yepiz-Plascencia et al., 1995). Lipoprotein subunits of *M borellii* HDL1 are within the wide range of molecular weight values reported for crustaceans (Lee, 1991), (Yepiz-Plascencia et al, 2000). The other plasma lipoprotein named HDL2 was found to be a vitellogenin (VG) that is present in hemolymph of females only during vitellogenesis. The lipid composition evidenced high content of sphingomyelin, contrasting HDL1, which allowed us to determine that these lipids are involved in the lipid-moiety order, thus the effect of a xenobiotic on HDL1 and HDL2 in *M. borellii* is differential (Garcia et al., 2002). HDL 1 and HDL 2 evidenced similar fatty acid composition, with 16:0 and 18:1-n9 (around 22%), 18:0 and 18:2 n-6 (around 12%), and 20:5 n-3, 18:1n-7 and 14:0 (less than 10%) (unpublished results).

LIPOVITELLINS AND VITELLOGENINS

In oviparous species, proteins, lipids and carbohydrates present in the vitellus are important sources of energy for the development of the embryo, and the growth and survival of larvae. Inside the eggs, lipids are distributed among membranes, cytosolic lipid droplets and lipoproteins. Membrane lipids have a structural function, and are mainly phospholipids and sterols of the embryo. Lipid droplets are composed of triacylglycerols and provide energetic fuels. Most proteins of invertebrate eggs are called vitellins (VT). High density

lipoproteins found in invertebrate eggs were named lipovitellins (LV) (Wallace et al., 1967), as well as the lipoproteins present in ovaries of vitellogenic females. Female-specific lipoproteins can be found in hemolymph during vitellogenic stage, and their structure may be similar or identical to lipovitellins. These sex-specific plasma lipoproteins are VG and are high density lipoproteins (HDL). Some authors named them LP 2 or HDL2, being its density higher than that of the sex-unspecific lipoprotein HDL1, also named LP 1. Decapod crustacean eggs contain LV of high or very high density (HDL or VHDL, respectively).

The anostraca *Artemia salina* has granule-shaped lipovitellins in their oocytes with relatively low lipid content (9 %). Unlike vertebrate lipovitellins, those of crustaceans do not have phosphorous bound to the apolipoproteins. Phospholipids are the predominant lipids, especially phosphatidylcholine, with fewer quantities of triacylglycerols and cholesterol, while cholesterol esters are generally absent or present in traces. Besides lipids and proteins, 3 to 4% of carbohydrates are found (de Chafoy and Kondo, 1980). Chen and Chen (1993) determined in *P. monodon* that LV was composed of four polypeptides of 168, 104, 83 and 74 kDa. The molecular weights of decapods native LVs generally range from 325 kDa to 500 kDa. The number of peptides and their molecular weights also vary among different species.

Lubzens and collaborators (1997) made experiments with anti-LV antiboides in *P. semisulcatus* and determined that LV and VG are composed of three apolipoproteins of 20, 120 and 80 kDa. Although LV synthesis site has arisen much controversy over the last years, they also determined that VG synthesis apparently occurs in the ovary and in a lesser extent in the hepatopancreas. The presence of an identical mRNA transcript of 1.0-1.1 Kb in both the hepatopancreas and ovary led the authors to suggest that both VG and LV are products of the same gen (Lubzens et al., 1995). On the other hand, Yano and Chinzei (1987) determined that VG is only synthetized in the ovary, based on in vitro studies of ovarian maturity in *P. japonnsicus* and with the use of anti-LV antibodies.

Based on antibody recognition and proteolitic mapping (Chen and Chen, 1993), it was concluded that VG of *P. monodon* had the same four polipeptides than that of the LV mentioned before. It was also confirmed that the subunits of 104 and 83 kDa of VG and LV are derived from another 168 kDa subunit present in these lipoproteins. In a subsequent paper, Chang and collaborators (1994) determined that VG of *P. monodon* was composed of subunits of 170 and 82 kDa. It had been previously determined in the same species that LV has more subunits that VG (Chang et al., 1993). From the

analysis of the aminoacid composition of VG and LV, it was determined that both of them were similar and it was suggested that VG is introduced within the oocytes and the larger subunits are cleaved to produce the smaller ones (Chang et al., 1994). Then it was shown the post-translational processing of *M. rosembergii´*s LV, through deductions of its primary structure (Okuno et al. 2002). Numerous studies were developed over the last years, an example being a study developed by Raviv et al. in 2006 where the complete sequence of *Lipopenaeus vannamei* VG mRNA was deduced. There have been considerable advances in this topic, so that VG receptors involved in vitellogenesis could be identified (Roth and Khalaila, 2012).

In the decapod *M borellii*, different studies on egg lipoproteins have been performed. LV characterization showed similarities between this egg lipoprotein and the VG HDL2 previously described. Both of them have in common their hydration densities (1.18 g/ml) and the mass of the native particle (440 kDa). Subunit determination by electrophoresis (94 and 112 kDa, respectively), as well as the use of an anti-LV polyclonal antibody, showed that the same lipoprotein particle is present in both lipoproteins (Garcia et al. 2008). More recently, correlations between the amount of VG/LV and ovary maturation have been reported ((Garcia et al., 2012). Also VG and LV were used as biomarkers for freshwater hydrocarbon pollution (Garcia et al., 2012).

Regarding lipid moiety, *M. borellii´s* VG and LV differ in their quantitative composition. Although both of them have similar total phospholipid contents (approximately 60%), LV doubles VG in its phosphatidylcholine content, has a slightly higher concentration of trialcylglycerols and has approximately half of the free fatty acid and cholesterol content than VG. This fact suggests the presence of structurally different lipid domains, and it also implies that the structural change poduced by a higher load of phosphatidylcholine and trialcylglycerols in LV possibly occurs in the ovary, in response of a functional necessity of the lipoprotein. A similar change was shown by Lubzens et al. in 1997. In this work they compared the lipid composition of VG and penaeid shrimp *Penaeus semisulcatus*, where trialcylglycerol content in LV was 2-fold higher than VG. Unlike *M borellii*, phospholipid percentage was unchanged.

We studied the conformation of *M. Borellii´*s LV by electron microscopy showing that its morphology was spheroidal (Garcia et al., 2008, Figure 5). These results are similar to insect plasma HDL (Gilbert and Chino, 1974; Pattnaik et al., 1979). In contrast, crustacean HDL1 was reported to be discoidal in lobster and crab (Lee and Puppione, 1978; Spaziani et al., 1986). For crustaceans, these authors determined that plasma HDL1 did not have

cholesterol esters but small amounts of triacylglycerols. In *M borellii* the spheroidal morphology of LV could be due to the presence of 20 % triacylglycerols (García et al., 2004), which can form a neutral lipid core (Garcia et al., 2008).

On the other hand, the lipoproteins called clottable proteins in *Peneaus vannamei* (Yepiz-Plascencia et al., 2002) and *Peneaus monodon* (Yeh et al., 1998) have structures formed by 200 kDa subunit homodimers. In *M. borellii´*s LV the protein particle seems to be a trimer, supported by the molecular weight of the cross-linked subunits (estimated in 320 kDa), and to the fact that the stain intensity of the smaller 94 kDa-subunit is about two-fold higher than the 112 kDa subunit (García et al., 2004). In this trimer the 112 kDa subunit seems to be more exposed to the aqueous environment, due to its high susceptibility to trypsine digestion in *in vitro* assays (Garcia et al., 2008).

Available information on the secondary structure of crustacean LVs is extremely scarce. Only the ovary LV of *P. vannamei* has been characterized, revealing a content of 37% β-sheets, 25 % α-helixes and 14 % turns (Garcia-Orosco et al., 2002). In contrast, in *M. borellii´*s LV we observed a greater content of α-helixes than that of β-sheets (35.7 and 16.6 %, respectively); which is similar to that observed by Yeh et al. (1999) in the clottable protein of *P. monodon.* It might be reflecting some important structural features for lipid binding and transport.

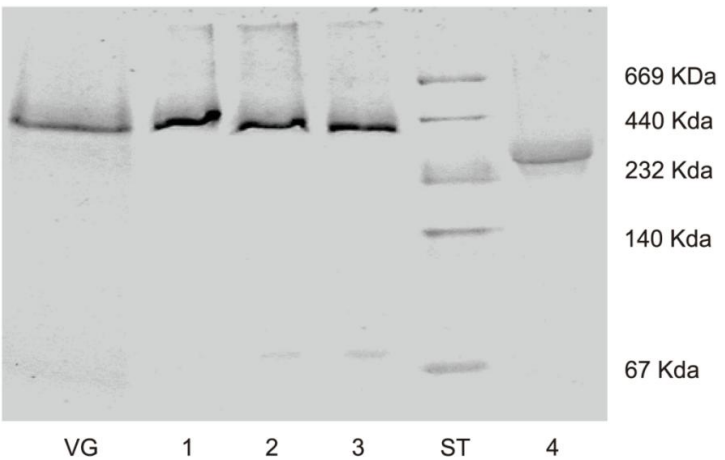

Figure 4. Native PAGE (4-23 % acrylamide gradient slab) of vitellogenin (VG) and lipovitellin isolated from three stages of embryo development: early (1), medium (2) and late (3); and HDL1 (4). ST: molecular weight standards.

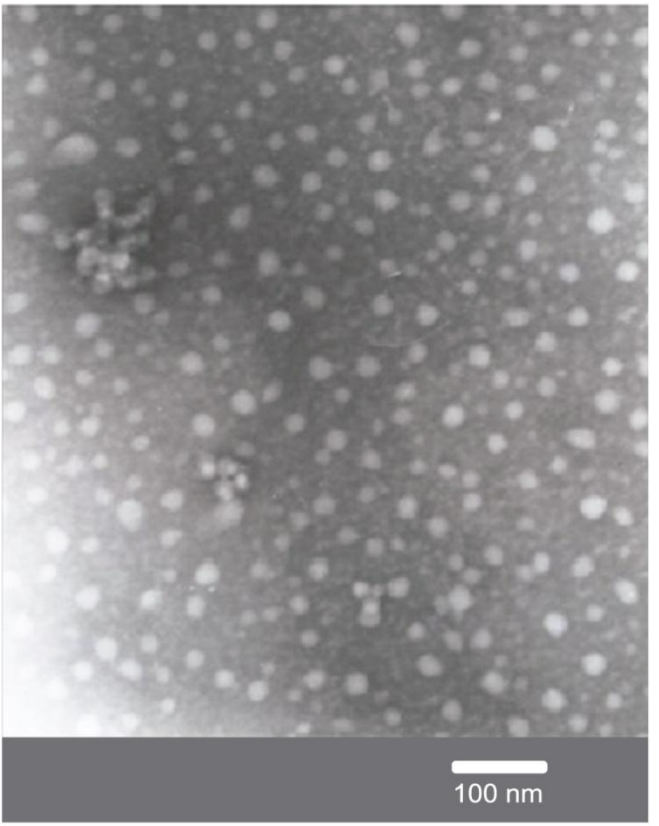

Figure 5. Electron micrographs of negatively stained lipovitellin isolated from *M. Borellii's* eggs. Final magnification X 120,000.

Regarding *M. borellii´*s LV fatty-acid composition, gaseous chromatoghraphy analysis showed that the major fatty acids were 16:0 (22 %), 18:1n-9 and 18:1n-7 (20.1 and 10.6 % respectively), 16:1 n-7 (14%), 18:1 n-7 (10.6%), and 18:0; the minor fatty acids were 18:2 n-6 and 20:5 n-3 with less than 10 % (Garcia et al., 2004).

LIPID TRANSFER BETWEEN HAEMOLYMPHATIC LIPOPROTEINS AND HEPATOPANCREAS

The hepatopancreas is the main lipid metabolic organ of crustaceans, it is very active for the synthesis as well as for the degradation of acylglycerols.

Liver and fat body are their analogue organs in vertebrates and insects, respectively. Hepatopancreas also performs digestive functions, enzyme secretion and excretes waste materials (O'Connor and Gilbert, '68; Al-Mohanna and Nott, '86). Also, as mentioned before, it is involved in lipoprotein assembly (Lee 1991).

In the year 2002 lipid transfer in crustaceans was determined by using the shrimp *M borellii* as a model. Lipid transfer was performed between the only lipoprotein present in the hemolymph of both male and female shrimp (HDL1) and the hepatopancreas in *in vivo* and *in vitro* assays using radiolabeled fatty acids injected into hemolymph. Fatty acid was rapidly incorporated into glycerolipid-synthesizing tissues, which subsequently released labelled lipids back to circulation. The increase of total circulating lipids was mainly produced by phosphatidylcholine and in lesser extent triacylglycerols and diacylglycerols (Figure 6). It was inferred that the phospholipid synthesis in tissues is more active than that of neutral acylglycerols. However, the radioactivity distribution among hepatopancreas lipids after the free fatty acid *in vivo* incorporation suggests that this organ is active for phosphatidylcholine synthesis at longer times, though at shorter times it is able to synthesize both phosphatidylcholine and acylglycerols. A similar observation was previously reported when we studied the lipid metabolism of *M. borellii* hepatopancreas (González Baró and Pollero, 1993). Also, the tissues are likely to retain the de-novo-synthesized triacylglycerols, transferring preferentially phosphatidylcholine to hemolymph. This fact is consistent with the results obtained from studies on the lipid composition of this and some other crustaceans, where phosphatidylcholine was found to be the predominant circulating lipid (Lee and Puppione, 1988), and triacylglycerols are accumulated in the hepatopancreas (González Baró and Pollero, 1988). The presence of the circulating label which was only found in the HDL fraction demonstrated the role of HDL1 in taking up lipids from tissues (Garcia et al 2002 Figure 7).

I*n vitro* studies on lipid transfer from hemolymph to hepatopancreas showed that hemolymph tends to supply the organ with free fatty acids as well as phosphatidylcholine. The transfer of free fatty acids corroborated previous observations, dealing with the components of the hemolymph-hepatopancreas system where hemolymph is likely to provide the fatty acids necessary for the synthesis of glycerolipids, task that was performed by a tissue of greater biosynthetic activity like the hepatopancreas. In other organisms, the release of the circulating free fatty acids to tissues is produced by high density lipoprotein structures which are poor in lipids. This is the case of the albumin-

free-fatty-acid complex in vertebrates or the very high density lipoproteins in certain molluscs (Heras and Pollero, 1990) and in insects (González et al., 1995).

In hepatopancreas labeled *in vivo* with [^{14}C]palmitate, radioactivity was mainly accumulated in phosphatidylcholine, and minorly in triacylglycerols and free fatty acids.

Labeled hepatopancreas were used for *in vitro* experiments and the transfer of these lipids to hemolymph was demonstrated. Free fatty acids were comparatively the most efficiently transferred lipids. Phosphatidylcholine, despite its highest labeling, showed a relatively lower transfer rate, whereas that of neutral acylglycerols was scarce. This also shows a markedly different behavior between glycerophospholipids and neutral acylglycerols, consistent with the large amount of phosphatidylcholine present in the circulating lipoprotein and an accumulation of triacylglycerols in the hepatopancreas under natural conditions.

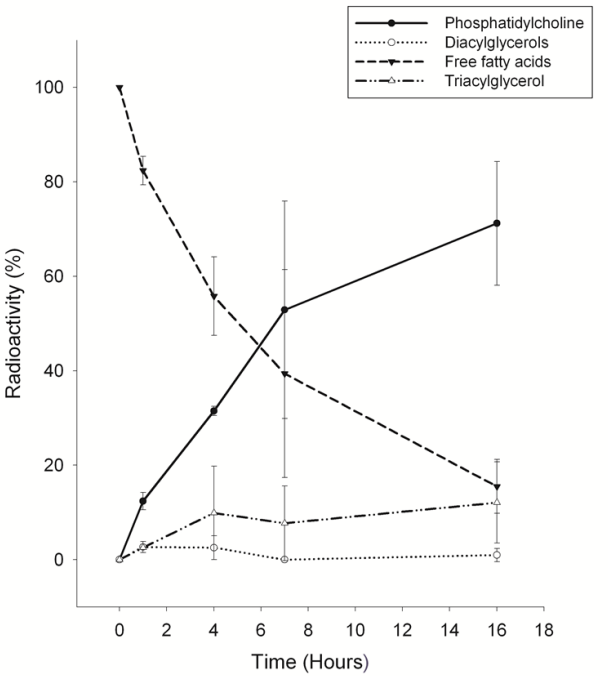

Figure 6. Distribution of radioactivity into lipid classes after injection of ^{14}C 16:0 of *M borellii* in hemolynph.

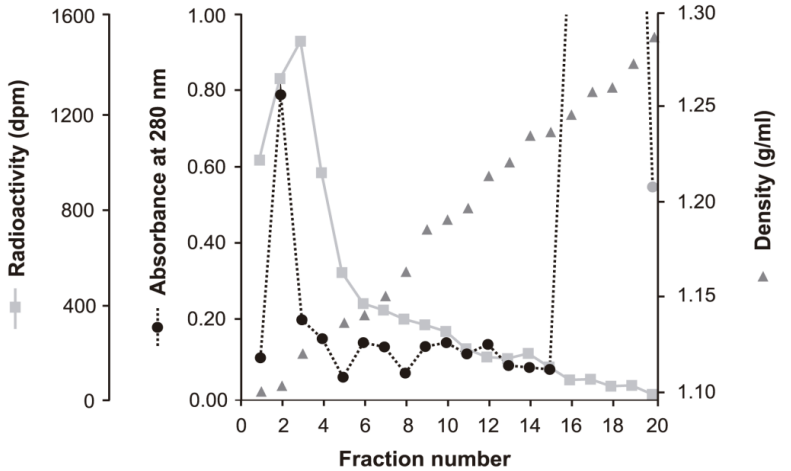

Figure 7. Protein distribution in plasma fractions of *M borellii* (•), absorbance at 280 nm, radioactivity (▫) and density (**Δ**). Shrimps were incubated *in vivo* with ^{14}C-palmitate.

LIPID TRANSFER FROM LIPOVITELLIN TO THE EMBRYO

*M borellii e*mbryogenesis was previously divided into seven stages (1 to 7) according to morphological features (Lavarias et al. 2002). During emrbyogenesis vitellus' lipids are the main energetic reserves followed by proteins. As mentioned before, lipids in the egg are structured in LVs. Although a lot of information on the origin of VG-LV is available, scarce studies were performed on LV degradation throughout development, which were restricted to crab *Callinectes sapidus (*Walker et al., 2003, 2006). In *M borellii* we analyzed LV in three stages of the embryonic development, early (stages 1, 2 and 3), medium (stages 4 and 5) and late (stages 6 and 7), and showed that both total lipids and proteins are consumed equivalently (Figure 8), consistent with the energy sources consumed in total vitellus (Heras et al., 2000).

Regarding LV lipids, phosphatidylcholine and triacylglycerol consumption was evidenced (Figure 9), being those the lipids accumulated in the LV from VG as mentioned before.

Although lipid transfer molecular mechanisms in tcrustaceans have not been described yet, the apolipoprotein present in both LV and HDL1 generates a possible lipid-protein interaction which implies an organizing effect of the

lipid phase (demonstrated by fluorescence spectroscopic measurements), similar to that described by Castuma and Brenner in 1989 and in Garda et al. 1994. This could suggest that an appropriate structure is necessary to generate a greater transference from phosphatidylcholine to hepatopancreas (Figure 10, Garcia et al. 2005). This issue was demonstrated by a study of *in vitro* phosphatidylcholine transference, comparing lipoprotein models (LV in early, medium stage and HDL1) and liposomes created with different lipids of each particle (Garcia et al. 2005).

By using anti-LV polyclonal antibodies we could analyze LV consumption throughout embryonic development estimated by ELISA (Garcia et al., 2008) shown in Figure 11, consistent with the depletion of lipids previously observed (Heras et al., 2000). In our study it was also determined that the subunit of 112 kDa was decomposed previously and throughout development, only remaining the subunit of 94 kDa. Proteolisis is more obvious in final stages 6 and 7. It is important to highlight that the presence of LV in embryos named LVe (LV of embryos) was reported. LVe showed immunological identity with LV-VG and shared not only its subunits but also its native molecular weight.

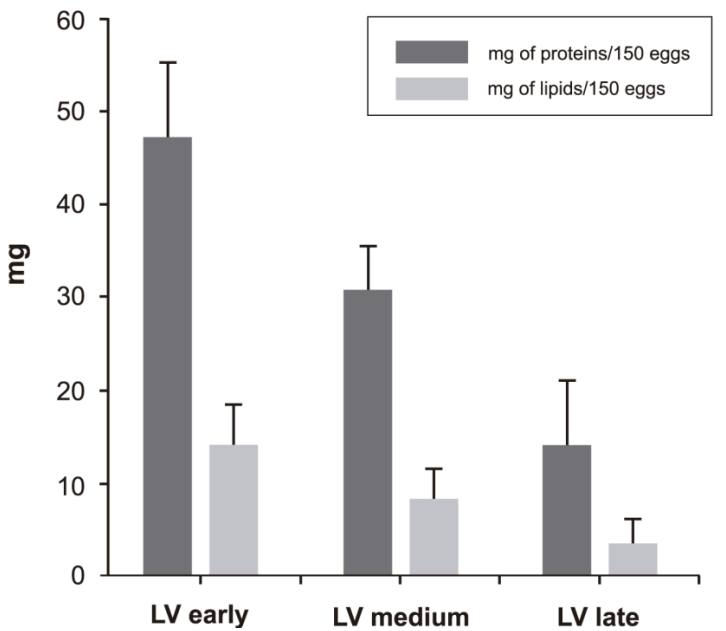

Figure 8. Lipid and protein quantitative changes of *M. borellii*'s lipovitellin along embryo development.

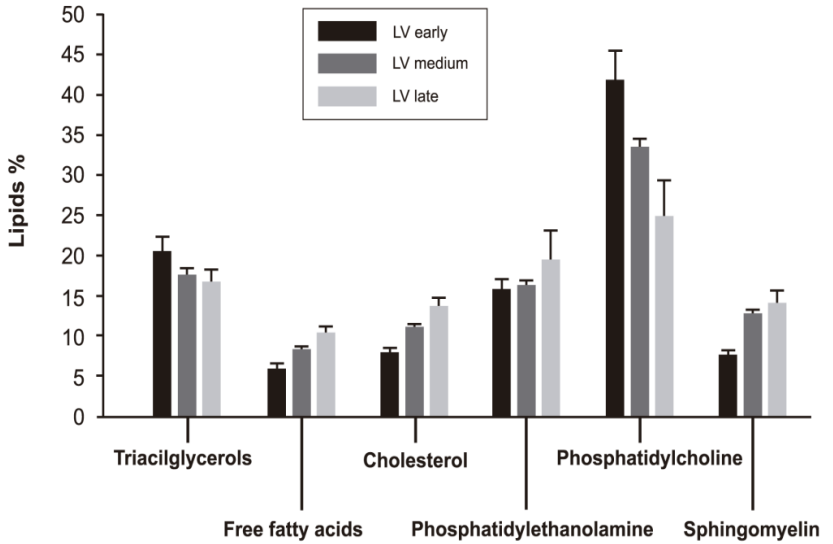

Figure 9. Lipid classes' changes of *M. borellii*'s lipovitellin along embryo development. (lipovitellin isolated from eggs at early, medium and late stages of embryo development).

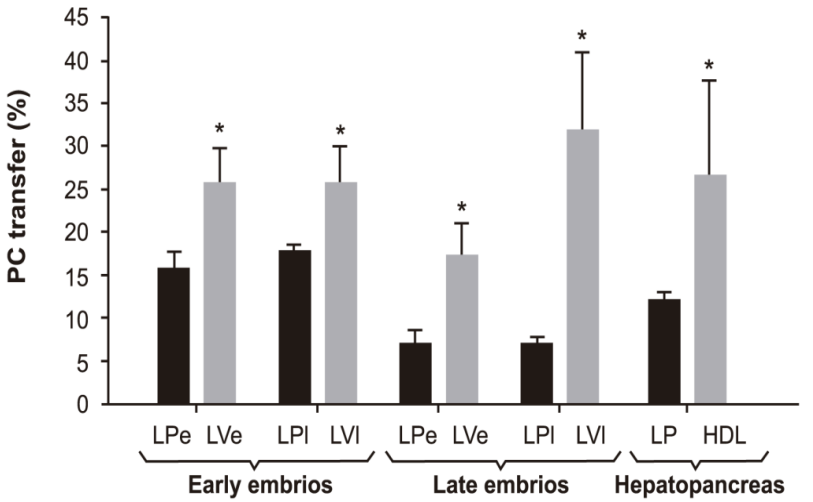

Figure 10. Percent of phosphatidylcholine transfer from lipoproteins (LV) and liposomes (LP) to tissues (early and late embryos, and hepatopancreas). Student´s *t* test was used to compare the significance of the differences between LVs and LPs: * $P<0.05$.

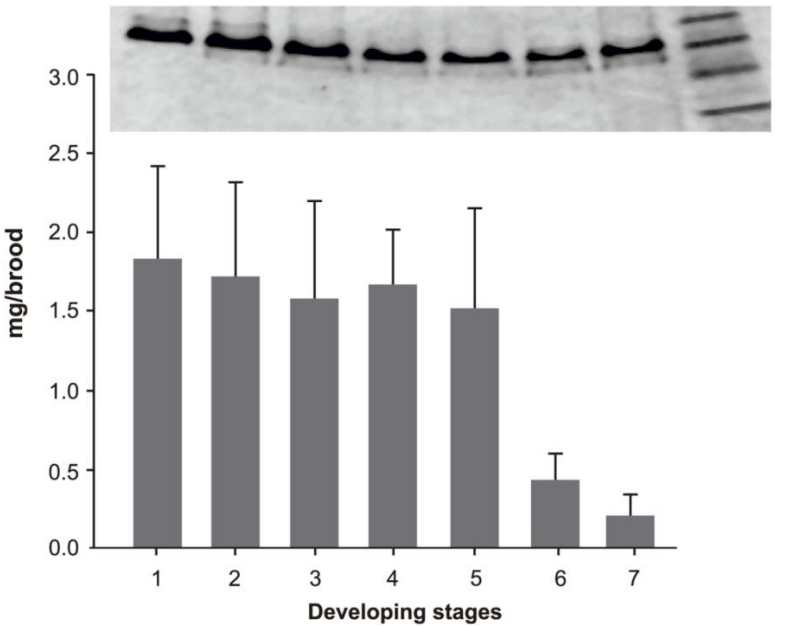

Figure 11. Changes of LV levels during *M borellii* embryogenesis. Polyacrylamide gel analysis of lipovitellins isolated from embryos.

Even though the knowledge of the lipid synthesis, transport and transference between organs is a very prominent field of study, further investigations are needed to bring light on the molecular mechanisms and regulation in crustaceans.

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Chapter 4

SOFT-BOTTOM CRUSTACEAN ASSEMBLAGES INFLUENCED BY ANTHROPOGENIC ACTIVITIES IN BAHRAIN, ARABIAN GULF

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ABSTRACT

The Arabian Gulf is a subtropical semi-enclosed sea characterized by marked fluctuations in sea temperatures and high salinities. Soft substrate benthos forms the largest and most diverse marine ecosystem.

Crustaceans constitute diverse and abundant taxonomic groups of soft-bottom macrofauna. These assemblages provide a useful tool for detecting environmental pollution and disturbance.

This study characterized soft-bottom crustacean assemblages influenced by anthropogenic activities in Bahrain in response to sewage-derived nutrients as well as industrial-derived hydrocarbons and heavy metals. Samples were collected subtidally from a small bay that receives sewage discharge from the largest plant in Bahrain, and off the eastern coastline that is influenced by effluents from the oil refinery and other major industrial factories.

Environmental parameters were synoptically measured, and nutrients as well as heavy metals were analyzed. Although crustaceans were

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severally impacted by localized sewage pollution, industrial effluents associated with hydrocarbons and heavy metals had more pronounced overall effects on crustacean assemblages represented by lower levels of diversity and abundance.

1. INTRODUCTION

The Arabian Gulf is a semi-enclosed sea situated in the subtropical zone and characterized by marked fluctuations in sea temperatures and high salinities. Sea surface temperatures below 15°C occurring in the winter and exceeding 35°C in the summer months are frequently recorded in the Arabian Gulf (Riegl and Purkis, 2012). Salinity can exceed 43 psu in the southern part of the Arabian Gulf and could reach 70-80 psu in areas with restricted flow such as tidal pools and lagoons due to high evaporation rates (Sheppard et al., 2010). Despite these harsh environmental conditions, the Arabian Gulf supports a range of coastal and marine habitats such as mangrove swamps, seagrass beds, coral reefs, and mud and sand flats (Naser, 2011a). These habitats provide feeding and nursery grounds for a variety of marine organisms, including a number of valuable commercial species (Carpenter et al. 1997).

Anthropogenically, the Arabian Gulf is considered among the highest impacted regions in the world (Halpern et al., 2008). Coastal development associated with intensive dredging and reclamation is increasingly contributing to the degradation of marine ecosystems (Naser, 2011b). Pollutants inputs affecting coastal and marine environments of the Arabian Gulf include domestic sewage, brine waste waters, and effluents from petroleum and petrochemical industries (Naser, 2011a).

The Arabian Gulf shallow sedimentary basin is mainly dominated by soft substrate benthic assemblages, which form the largest and most diverse marine ecosystem. Macrobenthos are largely composed of polychaetes, crustaceans, molluscs, and many other taxonomic groups. Crustaceans constitute diverse and abundant taxonomic groups of soft-bottom macrofauna (Ruso et al., 2007; Zainal et al., 2007; Lourido et al., 2008). They play an important role in structuring macrobenthic assemblages. Additionally, crustaceans widely contribute to trophic chains in marine ecosystems (Navaro-Barranco et al., 2012). Commercially, many species are considered important sources of food for humans (Abdulqader, 1999). Crustacean assemblages provide a useful tool for detecting environmental pollution and disturbance (Guerra-Garcia and

Garcia-Gomez, 2004). They may respond to environmental stresses by changes in their community structures represented by alterations in abundance, biomass and species diversity (Rinderhangen et al., 2000). Therefore, crustaceans have been used to detect environmental impacts from several sources of stress and pollution such as heavy metal contaminants (Blackmore et al., 1998; Farkas et al., 2003; Guerra-Garcia et al., 2010), oil spills and hydrocarbon pollution (Price et al., 1993; Gesteira and Dauvin, 2000; Nikitik and Robinson, 2003), sewage and nutrients enrichment (De-la-Ossa-Carretero et al., 2010, 2012a) and dredging activities (Robinson et al., 2005). Nonetheless, studies investigating the response of crustaceans to various sources of anthropogenic impacts in the Arabian Gulf have been limited (Naser, 2010a, b). The aim of this study therefore was to characterize soft-bottom crustacean assemblages influenced by anthropogenic activities in Bahrain in response to sewage-derived nutrients as well as industrial-derived hydrocarbons and heavy metals.

2. MATERIALS AND METHODS

2. 1. Study Areas

Tubli Bay and subtidal areas off the eastern coastline of Bahrain were selected. These areas are influenced by land-based anthropogenic activities including dredging and reclamation, sewage and industrial effluents, brine waste water discharges and oil pollution (Figure 1). The major sewage treatment plant in Bahrain (Figure 2) is discharging treated sewage, and partially treated when overloaded, into the shallow subtidal areas of Tubli Bay. The eastern coastline of Bahrain is heavily occupied by industrial facilities including the oil refinery (Figure 3), aluminum smelters and desalination plants (Figure 4). These industries are discharging effluents characterized by high inputs of heavy metals and hydrocarbons (Naser, 2011a, 2012).

2.2. Sampling and Parameter Measurements

Sediment sampling and environmental measurements were conducted in March, 2010. Ten stations in Tubli Bay and its equivalent off the eastern coastline of Bahrain were predetermined (Figure 5).

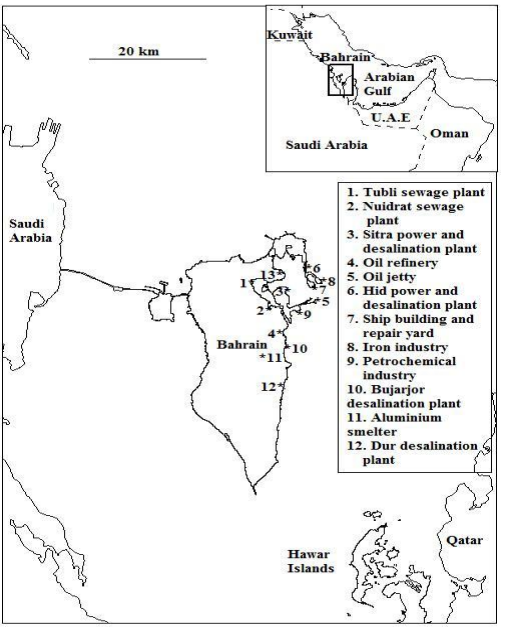

Figure 1. Map of major land-based anthropogenic activities influencing coastal and marine environments in Bahrain (Naser. 2012).

Figure 2. The outfall of Tubli Water Pollution Control Centre, the main sewage treatment plant in Bahrain.

Major impacts or features influencing the sampling stations in the study sites are summarized in Table 1. Environmental parameters including depth (m), alinity (psu), and water temperature (^{o}C) were recorded at each station. Seawater samples were collected from a depth of 20 cm, stored in a cold room at 0 ^{o}C for 24 h, and analyzed for nutrients; including ammonia, nitrate, and phosphate using a Palintest® photometer 8000 and proprietary assay kits.

Figure 3. Bahrain Oil Refinery discharges industrial effluents to the coastal and subtidal areas along the eastern coastline of Bahrain.

Figure 4. Sitra power and desalination plant discharges brine waste water that associated with high levels of salinity and temperature.

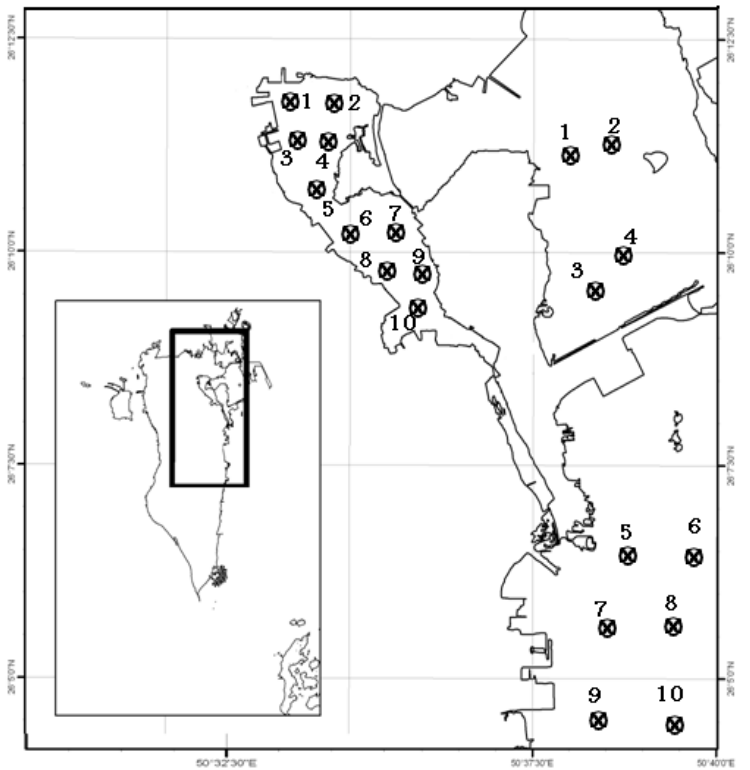

Figure 5. Approximate locations of sampling stations in Tubli Bay and off the eastern coastline in Bahrain.

Table 1. Major impacts/features influencing the sampling stations in the study sites

Site	Stations	Major impacts/features
Tubli Bay (T)	1, 2	Sewage discharge, reclamation
	3, 4	Sewage discharge, reclamation
	5, 6, 7	Reclamation
	8, 9, 10	Nearby mangrove swamps
Eastern coastline (E)	1	Brine discharge from desalination plant
	2	Navigational activities
	3	Industrial effluents
	4	Ship repairing dry dock
	5, 6, 7, 8	Effluents from the refinery, reclamation
	9, 10	Effluent from desalination plant

Faunal samples (triplicates) were collected using Van Veen grab (0.0675 m^2). An additional sediment sample was collected for grain size analysis as well as analysis of selected heavy metals.

Faunal samples were sieved in situ through a 0.5 mm mesh using seawater, fixed in 4% formalin, stained with drops of rose Bengal and subsequently preserved in 70% ethyl alcohol. Crustaceans were sorted according to their taxonomic groups, counted and identified to the lowest possible taxonomic resolution using relevant identification guides (Jones, 1986; Carpenter et al., 1997; Richmond, 2002).

2.3. Sediment Analyses

Sediment samples were analyzed physically (organic content and grain size analysis), and chemically (sediment digestion and analysis of the selected heavy metals). Sediment grain size analysis was conducted by sieving 50 g of homogenized sediment on a mechanical shaker through six sieves (mesh sizes 0.038-2 mm) and obtaining weight of sediment fraction in each sieve. The percentage of organic content in the sediment was obtained by incinerating a known weight at a temperature of 450 ^oC for 12 h.

Microwave-assisted digestion was conducted using a microwave (ETHOS D). The digestion was performed using 10 ml of nitric acid following to USEPA 3051 methods. The samples were analyzed for zinc, cadmium, lead, and copper using an atomic absorption spectrometer (A Analyst 100).

2.4. Statistical Treatments

Univariate and multivariate analyses were conducted using PRIMER® v6 (Clarke and Gorley 2006) to examine the biotic and abiotic differences between stations in Tubli Bay and those of the eastern coastline.

Ecological indices including diversity index of Shannon–Wiener, Margalef's index of richness and Pielou's index of evenness were calculated. Community structure and spatial variations of crustacean assemblages were analyzed by non-metric multidimensional scaling (nm-MDS) based on Bray–Curtis similarity index using square-root transformed abundance data.

3. RESULTS

3.1. Environmental Parameters

Subtidal waters surrounding Bahrain are relatively shallow. The average depth of sampling stations in Tubli Bay was 4.1 m (0.8-10.2 m) compared with 5.1m (1.1-12.3 m) for stations off the eastern coastline. Maximum depths were recorded at stations T2 and E2 which are near a navigational channel leading to a commercial port. Apart from station T1, which was composed of sludge (organic matter), most of the stations were characteristically described as fine sand.

Reduction in ambient salinity level (38 psu) was observed at station T1, which receives low salinity wastewater from the sewage plant. Conversely, an increase in salinity was measured in station E1, which is influenced by brine discharge from the power and desalination plant.

The measured sea temperatures in the study sites ranged between 24 and 26 ^{o}C in most of the stations. However, a substantial increase in sea temperature (37 ^{o}C) was recorded at station E1 at the proximity of the major power and desalination plants in Bahrain that produces brine wastewater effluents. The average concentrations of ammonia were 0.32 and 0.06 mgl^{-1} in Tubli Bay and off the eastern coastline, respectively. Station T1 that receives domestic sewage showed the highest value of ammonia (1.6 mgl^{-1}). Similarly, station T1 showed high levels of phosphate and nitrate concentrations (3.21 and 1.01 mgl^{-1}, respectively). Generally heavy metal levels in off the eastern coastline were higher than in Tubli Bay, with the exception on zinc. The mean concentrations of zinc, cadmium, lead, and copper in Tubli Bay were 48.6±12.3, 0.98±0.51, 13.1±2.3 and 12.4±5.6 $mgkg^{-1}$, respectively, compared with 39.5±15.4, 1.75±1.1, 21.9±5.3, 16.2±4.8 $mgkg^{-1}$ off the eastern coastline, respectively. The high levels of metals off the eastern coastline might be attributed to the industrial effluents from major factories and plants located in the industrialized coastal zone.

3.2. Crustacean Community Structure

In total, 1044 crustacean individuals belonging to 18 species and eight orders were collected from the study sites. Decapoda was the best represented taxon with seven species followed by Amphipoda and Isopoda with four and two species, respectively.

Table 2. Ecological indices of crustacean assemblages collected from stations in Tubli Bay and off the eastern coastline of Bahrain

	Tubli Bay										Eastern coastline									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Number of species	0	3	4	4	5	5	5	8	8	5	5	3	3	4	1	2	2	3	3	5
Number of organisms $0.02m^{-2}$	0	64	134	79	54	48	43	225	75	97	54	72	14	14	2	5	10	10	8	36
Species richness	-	0.48	0.16	0.68	1.00	1.03	1.06	1.29	1.62	0.87	1.00	0.47	0.76	1.3	0	0.62	0.43	0.68	0.96	1.12
Species evenness J'	-	0.66	0.35	0.54	0.73	0.71	0.76	0.25	0.70	0.42	0.83	0.18	0.91	0.80	0	0.97	1.00	0.58	0.81	0.60
Diversity H'(loge)	-	0.73	0.48	0.75	1.17	1.15	1.22	0.52	1.45	0.67	1.34	0.19	1.00	1.11	0	0.67	0.69	0.64	0.90	0.96

Tanaidacea, Cumacea, Mysida, Leptostraca and Sessilia orders were represented by one species each. Ecological indices of crustacean assemblages collected from the study sites are presented in Table 2.

Tubli Bay showed higher levels of both diversity and abundance of crustacean assemblages than those off the eastern coastline. A total of 819 individual crustaceans belonging to 15 species were collected from Tubli Bay compared with 225 individuals belonging to 11 species in areas off the eastern coastline. In Tubli Bay, amphipods were numerically the abundant group representing 78% of the total crustacean population (Figure 6). Sampling areas off the eastern coastline were dominated by amphipods and tanaidaceans, which accounted for 45% and 39%, respectively (Figure 7).

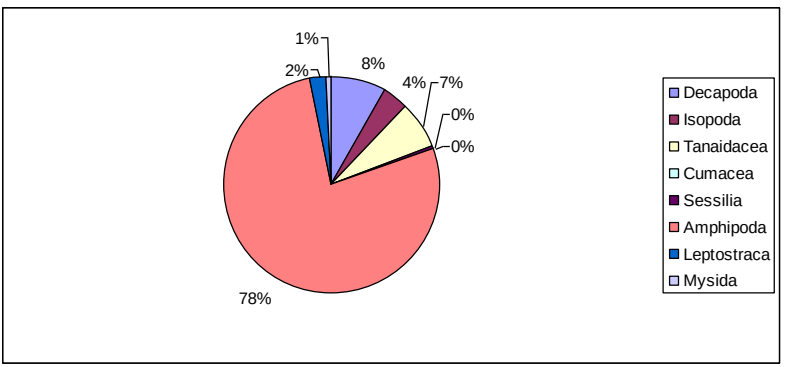

Figure 6. Numerical dominance of crustacean groups in Tubli Bay (n = 819).

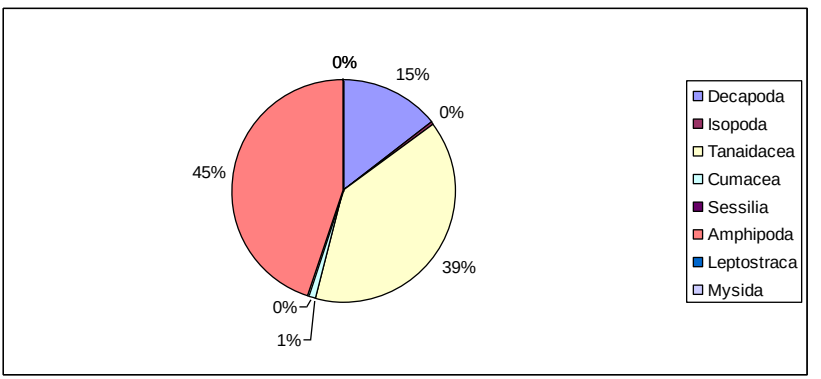

Figure 7. Numerical dominance of crustacean groups of the eastern coastline (n = 225).

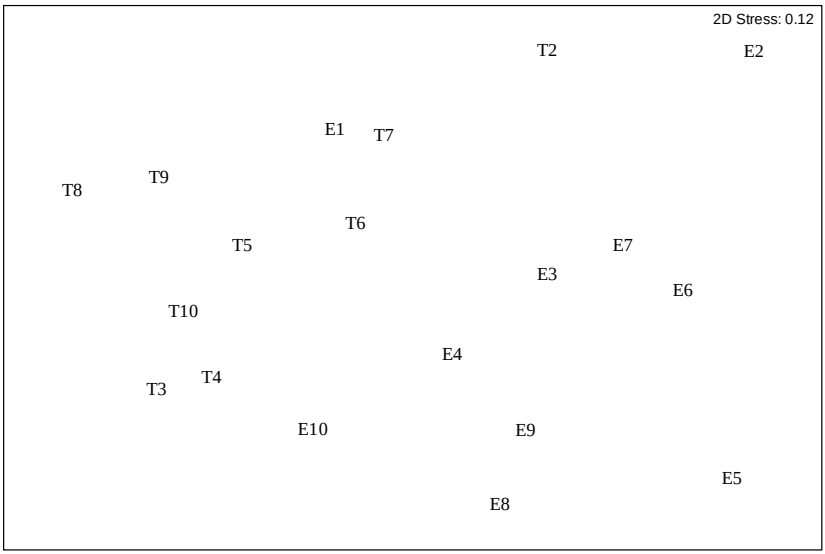

Figure 8. MDS plot for square-root transformed crustacean abundance using Bray–Curtis similarity coefficient.

The MDS of crustacean abundance revealed a clear separation between samples collected from the Tubli bay and eastern coastline site (Figure 8) suggesting pronounced differences in their community structure.

4. DISCUSSION

Crustaceans are abundant and widely distributed over a variety of aquatic systems (Bundschuh et al., 2011). Crustaceans represent a major source of food for several marine organisms and fishes of commercial importance. Additionally, they significantly contribute to the overall benthic production in marine ecosystems (Rinderhangen et al., 2000). Environmental changes such as biochemical composition of sediment, organic content, and pollutants can alter the distribution and abundance of crustaceans (Sanchez-Moyano and Garcia-Asencio, 2010). It is commonly reported that crustaceans are usually more sensitive to pollution than other benthic taxa (Dauvin and Ruellet, 2007). Therefore, crustaceans are intensively used as bioindicators and biomonitors to detect environmental stresses and pollution in coastal and marine environments (Rinderhangen et al., 2000). Structures of crustacean

assemblages, including biodiversity and abundance can provide information about the environmental state.

Generally, results of the present study indicated that sewage, and industrial effluents had pronounced effects of crustacean assemblages represented by lower levels of biodiversity and abundance. Crustaceans are sensitive to sewage pollution (De-la-Ossa-Carretero et al., 2010; Ates et al., 2011). Several studies reported a decreased abundance of amphipods nearby sewage outfalls, which is considered an indicator for sewage pollution (De-la-Ossa-Carretero et al., 2012a, b). Station (T1) at the proximity of the outlet of the major sewage plant in Bahrain was devoid of crustaceans reflecting severe sewage pollution in that area. This station was composed of sludge due to the continuous discharge of domestic sewage to the shallow water of Tubli Bay. Sewage discharges can change the organic contents in both the water column and sediment and subsequently affect associated marine organisms (Bundschuh et al., 2011).

Mangrove ecosystems form important nursery and feeding grounds for many crustacean species (Lee, 2008). Recognizing the critical condition of mangrove ecosystems, Bahrain declared the mangrove area in Tubli Bay as a natural protected site in 1995, and as a wetland of international importance (Ramsar Convention of Wetlands) in 1997. However, due to the current lack of management plans for the protected areas (Al-Sayed et al., 2008), Tubli Bay and its associated mangrove stands are severely subjected to human impacts that might affect the existence of mangrove ecosystems. The marine area of this bay has been reduced substantially from 25 to 12 km^2 in 2008 as a result of reclamation activities, which subsequently reduced and destroyed mangrove stands. Presently, the estimated cover of mangrove plants is 0.31 km^2 (Abido et al., 2011). Despite the continuous human-induced pressures on mangrove habitats in Bahrain, they play an important role in maintaining diversity and productivity of marine benthos, including crustaceans (Grabe et al., 2004). Stations at the proximity of mangrove plants in Tubli Bay showed high levels of crustacean diversity and abundance. For instance, around 27% of crustacean population in Tubli Bay was collected from station T8, which is located close to the main mangrove swamp.

Hydrocarbon and heavy metal contaminates are severally affecting the structure of the crustacean community (Gesteira and Dauvin, 2000; Nikitik and Robinson, 2003; Marsden and Rainbow, 2004; Lee et al., 2005). The eastern coastline of Bahrain is recognized as an area with high inputs of petroleum hydrocarbons (De Mora et al., 2010) and heavy metals (De Mora et al., 2005; Naser, 2012) from a wide range of industrial activities. Results of

the present study indicated that most of the analyzed heavy metals exhibited higher levels of concentrations in sediments collected from stations off the eastern coastline. Cumulative effects of industrial effluents that contain high levels pollutants, brine discharges, and sedimentation due to intensive dredging and reclamation activities on the eastern coastal areas were reflected on the reduced levels of crustacean diversity and abundance. Numerically, crustaceans in Tubli Bay accounted for 78.4 % of total population compared with 21.6% for those off the eastern coastline of Bahrain.

This pronounced difference in the structure crustacean assemblages is reflected in the separation between stations of the two areas on the MDS (Figure 8). Similarly, reduced levels of crustacean diversity and abundance in areas off the eastern coastline in Bahrain were reported by Al-Wedaei et al. (2011).

They reported that crustaceans only accounted for 5% of the total benthic community population in the eastern coastline compared with 39% in the western coastline of Bahrain. This reduction in crustacean assemblages was primarily attributed to the overall environmental degradation in areas off the eastern coastline (Al-Wedaei et al., 2011).

Crustaceans are significantly contributing to the overall productivity of coastal and marine ecosystems in the Arabian Gulf (Grabe et al., 2004; Al-Maslamani et al., 2007; Al-Yamani and Khvorov, 2007).

However, the Arabian Gulf is witnessing a rapid coastal development that is associated with biological, chemical and physical changes in coastal and marine environments, which may consequently affect the community structure of crustacean assemblages (Naser, 2010b). Naser (2011a) investigated studies conducted on macrobenthos in the Arabian Gulf and reported a general trend of reduction in diversity and abundance of these organisms, including crustaceans.

This was attributed to the environmental degradation due to escalated anthropogenic activities in the Arabian Gulf (Naser, 2011a). Similarly, low numbers of diversity and abundance of crustaceans in Environmental Impact Assessment (EIA) reports of major development in the coastal and marine environments in Bahrain were observed by Naser et al. (2008). The notable degradation in crustacean assemblages (lower biodiversity and abundance) in Bahrain is generally attributed to sedimentation due to dredging and reclamation and pollution from a variety of land-based activities (Naser, 2010b, 2011a).

CONCLUSION

Domestic sewage, industrial effluents from petroleum and petrochemical industries, brine waste water discharges, and reclamation activities are major sources that contribute to the biological degradation of marine organisms, including crustacean assemblages. Crustaceans are ecologically-sensitive organisms and effective indicators for natural and disturbed environmental conditions.

This study showed that investigating community structures of crustaceans represented by species composition and abundances can reflect the anthropogenic state of the environment. Crustaceans in the Arabian Gulf play an important role in structuring benthic assemblages and contribute to the overall productivity of marine ecosystems. Therefore, monitoring of crustacean assemblages should be an integral part of any conservation strategy in order to preserve the marine environments and the commercially important species they support.

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