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Heavy metal accumulation and its impact on structural and biochemical changes in the lobster *Panulirus homarus homarus* (Linnaeus, 1758)

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Original Article

Abstract

Distribution of heavy metals and its associated structural and biochemical perturbations were studied in the tissues of lobster *Panulirus homarus homarus* collected from Royapuram harbour which receives effluents from various industries located in northern coast of Chennai and compared with that of the less polluted Kovalam coast. The concentrations of copper, zinc, iron and cadmium were quantified in gills, muscles, hepatopancreas and gonadal tissues. The results showed marked differences between the two sites as well as significant variations were observed between the tissues. Metal accumulation was seen in the order of Cu> Zn >Fe>Cd in lobsters collected from Royapuram (polluted site) and Zn>Cu>Fe>Cd from Kovalam (less polluted site). Overall, the highest metal concentrations were found in the lobster collected from Royapuram coast. Of various tissues, the metal accumulation was high in hepatopancreas and ovary of *P. homarus homarus*. Structural architecture of the tissues was further investigated by light and scanning electron microscopy. Histological studies clearly indicated degeneration and atrophy of muscle fibres, lumen disruption in hepatopancreas, conspicuously ovary showed mature oocytes without yolk granules and disruption of follicle cell in lobsters collected from Royapuram coast. However, such structural deformities were not encountered in lobsters from Kovalam. Similarly scanning electron micrograph of gill showed normal surface topography with prominent gill filaments in lobsters collected from Kovalam, whereas, gill architecture was completely disrupted in lobsters from Royapuram. This clearly reflects the impact of metal pollution on various tissues of lobsters, which may affect lobster resources in east coast of India.

Keywords: *Panulirus homarus homarus, industrial effluents, heavy metals, atrophy, necrosis*

Introduction

The natural aquatic systems may extensively be contaminated with heavy metals released from domestic, industrial and other man-made activities and they cause serious threats because of their toxicity, long persistence, as well as bioaccumulation and biomagnifications in the food chain (Kamaruzzaman *et al.*, 2007). Metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene and Jankaitem, 2006; Farombi *et al.*, 2007). Thus, determination of harmful and toxic substances in water, sediments and biota will give direct information on the significance of pollution in the aquatic environment (Hugget *et al.*, 1973).

Furthermore, there are several factors influencing the

elimination of metals from the tissues of aquatic animals (Skejelkvale *et al*., 2001). These include period of exposure, surrounding temperature, interacting agents, age and the metabolic activity of the animal, and biological half life of metals. Metal elimination routes are more numerous than uptake routes; however, metal accumulation is more rapid than its elimination, and this is probably due to the presence of metal binding proteins in the tissues (Soegianto *et al*., 1999). Benthic invertebrates show considerable potential as sentinel marker species for ecosystem health monitoring programmes because they are small, common and relatively sessile and they tend to accumulate toxicants present in the environment. In addition, the biochemical, physiological and histological characteristics of several common species are sufficiently well known to distinguish exposed individuals from non-exposed individuals (Viarengo, 1993). Crustaceans can accumulate metals in their systems via absorption from the surrounding water or sediment or ingestion of food (Bryan, 1979). The need for monitoring environmental conditions in relation to the trace metal contamination of aquatic systems has resulted in the development of bio-indicators for this complex task (Phillips, 1977; Luoma, 1983). The interest in obtaining an accurate and easy analysis of the trace elements from marine crustacean tissues arises from nutritional, toxicological and environmental perspective.

Nevertheless, to our knowledge, with reference to biochemical and physiological aspects of crustacean tissues, only very scanty reports are available on the architecture and ultrastructure of these tissues. Therefore, this study is attempted to document the possible accumulation of metals in vital tissues of lobster *Panulirus homarus homarus*. The lobster *P. homarus homarus* being benthic in nature and those in Royapuram are exposed to heavy metal concentrations due to industrial pollution, domestic sewage and drastic changes in environmental conditions, were selected and compared against those in the less polluted Kovalam coast, free from anthropogenic inputs.

Material and methods

Sampling Site: Royapuram (13.1040° N, 80.2937°E), located in the northern part of Chennai city is one of the most important place involving in a wide range of anthropogenic activities and surrounded by various industries. There are about 572 fishing crafts and more than 1375 other boats harboured, polluting the sea extensively. The reference site used for the study is Kovalam coast (12° 49'N, 80° 5'E) which is free from anthropogenic inputs, located 40 km south of Chennai. It runs parallel to the sea coast and extends 20 km inland.

Sample collection and processing: Twenty specimens of *P. homarus* were collected from the landing centers in the study area and immediately brought to the laboratory in insulated boxes filled with ice. Carapace length was measured and the sex was identified. Tissue samples including gills, hepatopancreas, muscles, testis and ovary were taken from each lobster for further analysis.

Metal analysis: Samples of gills, hepatopancreas, muscle, testis and ovary were dissected, washed with distilled water, weighed, packed in polyethylene bags and stored at -20°C. To prevent metal contamination of the samples by the laboratory equipments, special care was taken and tissues were dissected by plastic knife and all laboratory wares were soaked in 2M HNO_3 for 48 hr, and rinsed with distilled water, and then with deionized water prior to use. Frozen biota samples were thawed at room temperature and dissected using stainless steel scalpels. Known quantities of the samples were oven dried at 90°C for 24 hr. After complete dryness the tissues were homogenized in mortar and pestle separately. The dried powder tissue samples were then weighed accurately to approximately 2g. The samples were transferred to a 25 ml conical flask, to which10 ml of 4:1(v/v) nitric acid and perchloric acid mixture were added. Each conical flask was then covered with a watch glass and allowed to stand overnight at room temperature. Then the samples were digested to near dryness by evaporating liquid at 90°C on a hot plate and cooled to room temperature. The digested samples were then filtered through Whatman no.1filter papers and collected in the 50ml beakers. The filters were rinsed thoroughly with deionized water. Contents of the beakers were quantitatively transferred to the 10 ml volumetric flasks, brought to volume with double distilled deionized water. Element contents in the samples were analysed by atomic absorption spectrometer (Perkin-Elmer, AA700)

Biochemical analysis: Protein content in various tissue samples of lobsters collected from both Kovalam and Royapuram coast was determined according to the standard procedure of Bradford (1976). Similarly the carbohydrate and lipid content were determined according to Carroll *et al.* (1956) and Folch *et al.* (1957) respectively.

Histology: The organs such as gill, hepatopancreas, muscle, testis and ovary were quickly removed and fixed in 10% neutral buffered formaldehyde solution (pH 7.0). After fixation (minimum time 18-24 hr); tissues were dehydrated in graded alcohol series and embedded in paraffin wax. Fine sections of 6 μm thickness were taken, stained with haematoxyline and eosin (HE), and observed under Leica 2500 microscope.

Scanning electron microscopy: Gill tissue was acquired by dissection through the full thickness of the central regions of each gill from each specimen. This was placed in fresh 2%

glutaraldehyde in filtered sea water at pH 7.2 for 1hr. The tissue was further washed in fresh sea water, post-fixed in a solution of 1% osmium tetroxide in sea water (pH 7.2), dehydrated through graded ethanol series and critical point dried using carbon-di-oxide. The specimen was trimmed and mounted on brass stubs using carbon tape and individually oriented to show either filament inter-lamellar connections, the frontal or abfrontal aspects of rows of filaments or the lateral aspects of individual filaments. Each stub was sputter-coated with approximately 10 nm of gold using a Polaron SC500 coater; Specimens were then examined using a JEOL 6100 Scanning Electron Microscope (SEM) at accelerating voltage of 10 to 15 KV.

Statistical analysis: Analysis of variance (ANOVA) was performed to determine if there is any significant difference of metals between the tissues of lobsters collected from Kovalam and Royapuram. This was performed using the commercial software package SPSS (version 11.2).

Results

Bio accumulation: Heavy metal accumulation in various tissues of *P. homarus homarus* showed significant variations (Figs. 1-4). The gills of the lobster collected from Royapuram coast, showed the highest concentration of Iron (12.45 ± 2.45 μg g^{-1}), followed by copper (6.28 ± 1.03 μg g^{-1}), zinc (4.89 ± 0.98 μg g $^{-1}$) and cadmium (0.35 ± 0.09 μg g^{-1}). The hepatopancreas registered maximum of 20.79 ± 3.28 μg Cu g^{-1}), 16.83 ± 3.7 μg Zn g^{-1}), 6.22 ± 1.57 μg Fe g^{-1}and 0.74 ± 0.16 μg Cd g^{-1}). The concentrations of zinc, copper, iron and cadmium in ovary were 12.45 ± 2.06 μg g^{-1}), 9.38 ± 1.99 μg g $^{-1}$), 7.37 ± 1.58 μg g $^{-1}$), 0.51 ± 0.12 μg g^{-1}) respectively. In testis the metal accumulation pattern was seen in the order of zinc (8.84 ± 1.99 μg g $^{-1}$) followed by copper (5.55 ± 0.09 μg g^{-1}), iron (4.93 ± 0.90 μg g^{-1}) and cadmium (0.27 ± 0.04 μg g^{-1}). However, muscle tissue showed the lowest accumulation of metals with 7.93 ± 1.57 μg Zn g^{-1}), 4.23 ± 0. 96 μg Fe g^{-1}), 2.36 ± 0.27 μg Cu g^{-1}) and 0.21 ± 0.03 μg Cd g^{-1}).

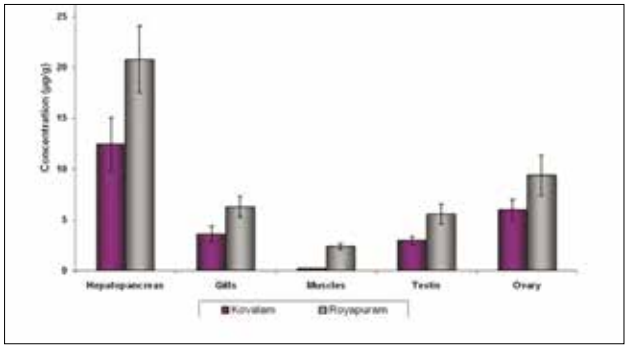

Fig. 1. Copper concentration (μg g^{-1}) in the tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

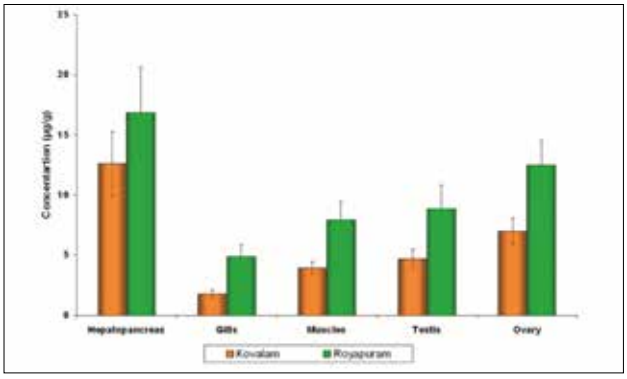

Fig. 2. Zinc concentration (μg g^{-1}) in the tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

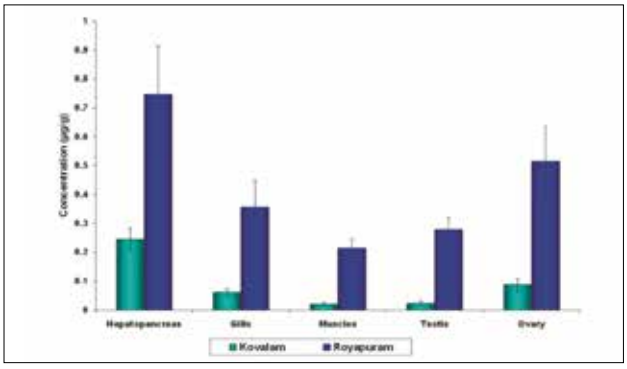

Fig. 3. Cadmium concentration (μg g^{-1}) in the tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

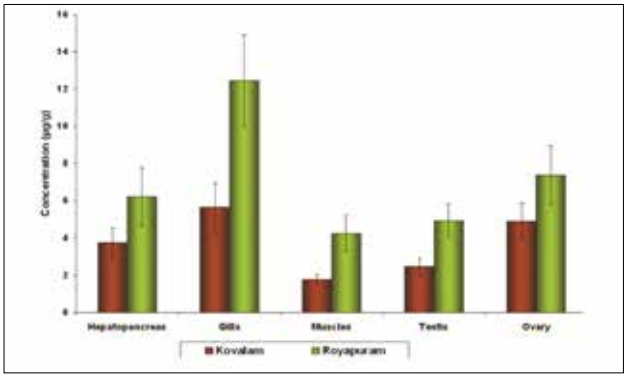

Fig. 4. Iron concentration (μg g^{-1}) in the soft tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

On the other hand, the lobsters collected from the Kovalam coast registered maximum levels of metals in the hepatopancreas (12. 61 ± 2.68 μg Zn g^{-1}), 12.46 ± 2.58 μg Cu g^{-1}) and 0.24 ± 0.03 μg Cd g^{-1}) except for iron which was observed at higher concentration in gills (5.64 ± 1.22 μg g^{-1}). Lowest concentration of metals were observed in the muscles with 3.94 ± 0.52 μg g^{-1} of zinc, 1.77 ± 0.27μg g^{-1} of iron,0.18 ± 0.03μg g^{-1} of copper and 0.08 ± 0.02 μg g^{-1} of

cadmium. Overall, the accumulation of copper, zinc, iron and cadmium were high in the lobster collected from Royapuram coast. The results demonstrate that the concentrations of copper, zinc and cadmium were higher in the hepatopancreas, whereas, maximum iron concentration was observed in gills and significantly lower in muscles collected from both landing centers. Metal accumulation varied significantly in tissues of lobsters between two sites ($p<0.05$).

Biochemical variations: The variations in protein, lipid and carbohydrate in different tissues are presented in figs. 5-7. The protein content registered maximum in hepatopancreas (62. 73 ± 23.55 μg/mg) followed by muscle (56. 49 ± 24.65 μg/mg) and minimum concentration in gills (46.14 ± 22.4 μg/mg) from the lobsters collected from the Kovalam coast. Whereas in the polluted site maximum protein content was recorded in muscles 37.26 ± 10.41 μg/mg and minimum in gills (23. 80 ± 12. 85 μg/mg). Lipid (35.7± 26.94 μg/mg) and carbohydrate (11.41 ± 1.15 μg/mg) were high in hepatopancreas, whereas minimum lipid (20.56 ± 23.13 μg/mg) and carbohydrate (5.46 ± 0.83 μg/mg) were observed in testis and ovary respectively in *P. homarus homarus* collected from Kovalam. In polluted site, the lipid content decreased to 18.46 ± 18.46 μg/mg in ovary and to (11.22 ± 12.55 μg/mg) in gills. In contrast, carbohydrate content (7.54 ± 1.02 μg/mg) registered maximum in hepatopancreas and minimum in ovary (20.56 ± 23.13 μg/mg). Overall protein, lipid and carbohydrate content substantially decreased in the lobsters collected from Royapuram coast.

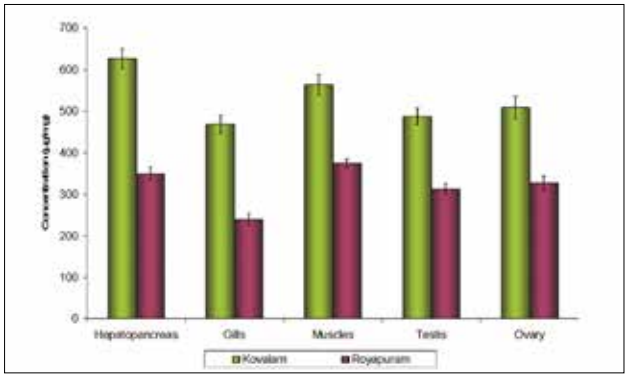

Fig. 5. Total protein (μg mg^{-1}) content in various tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

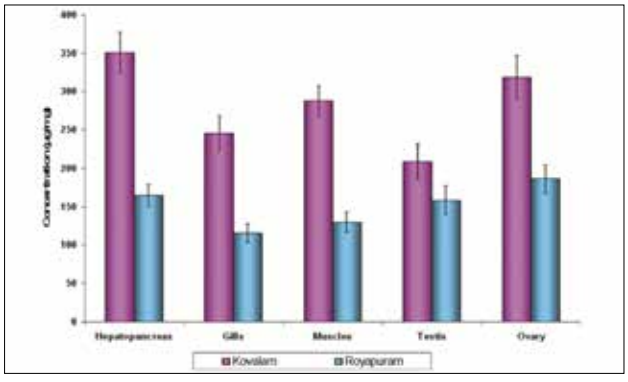

Fig. 6. Lipid content (μg mg^{-1}) in various tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.055$

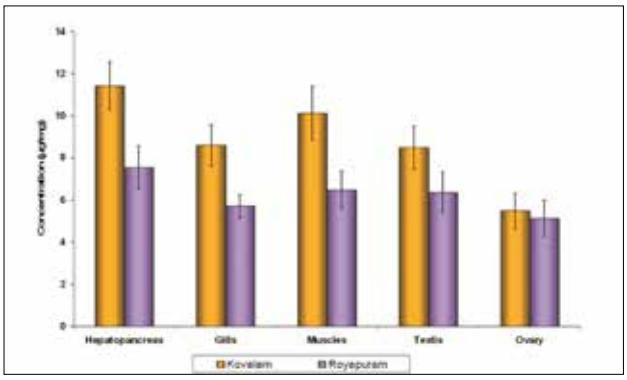

Fig. 7. Carbohydrate content (μg mg^{-1}) in various tissues of lobster collected from Kovalam and Royapuram coast. X ± SD of three observations. *F-test $p < 0.05$

Histological changes in different tissues

Gills: The section through the gills of lobster collected from less polluted site (Kovalam) revealed normal architecture, such as uniform thin cuticle and a series of primary and secondary gill filaments in which plaster cells, marginal canal and ionocytes were marked cleary and were well connected by connective tissues (Figs.8a and b). Whereas, the gill of lobster collected from Royapuram (polluted site) showed irregular arrangements of gill filaments with distorted cuticle and varying inter filamentary spaces. Within the secondary filament, necrosis and connective

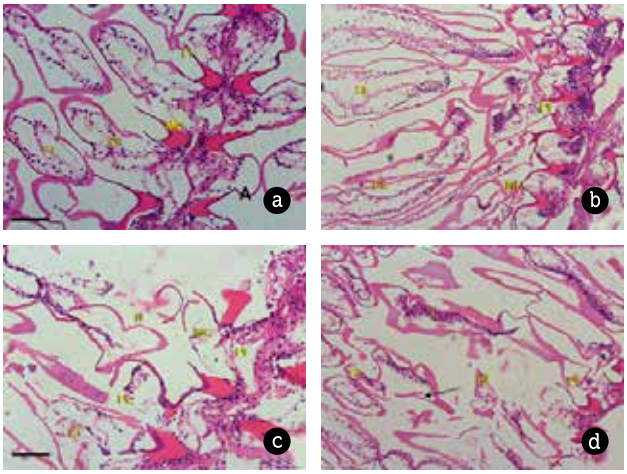

Fig. 8. (a–d) Photomicrographs of gills of *P. homarus.* (Figs. a and b) Lobster collected from Kovalam showing normal arrangement of cuticle structure

tissue damages were observed (Fig. 8c). The primary filament was detached from the cuticle and the ionocytes become invaded from the secondary gill filament. Some of the secondary gill filaments were inflamed (Fig. 8d). Over all the entire lamellae seem to be collapsed and hyperplasia was also observed.

Muscle: The architecture of muscle collected from Kovalam showed customary arrangements of muscle bundles and muscle fibers, binding to connective tissue. The striated muscle fibers were tightly packed (Figs. 9a and b). In contrast, muscles of lobster collected from Royapuram was completely disrupted and showed atrophy in the muscle bundles (Fig. 9 c). Splitting of muscle fibers was seen along with complete degeneration of connective tissues. Conspicuous vacuoles were also observed in between the muscle fibers (Fig. 9 d).

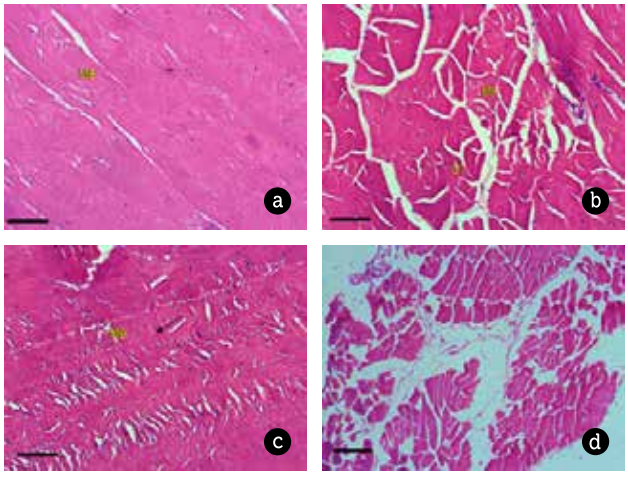

Fig. 9. (a–d) Sections through the muscle of *P. homarus.* (Figs. a and b) Lobster collected from Kovalam showing normal arrangement of muscle fiber and muscle bundles

Hepatopancreas: Lobsters, collected from the reference site showed densely packed hepatopancreatic tubules, and inter tubular spaces were joined by a thin layer of connective tissue that most likely contains blood vessels. The tubular tissues were composed of a coloumnar epithelium delimited at its base by a basement membrane and characterized at its distal end by a striated border (Figs. 10 a and b). However, the hepatopancreas in lobsters collected from the polluted site showed necrotic tubules that contained tissue debris in the lumen. Moreover, there appeared to be a severe walling off from the tubules by hemocytes around the thickened basal laminae. Internal organization of the tubule and the lumen was disrupted and the connective tissue appeared to be completely loose (Figs. 10 c and d).

Testis: Sections through the testis of the lobster collected from Kovalam coast exhibited sub-spherical follicles filled

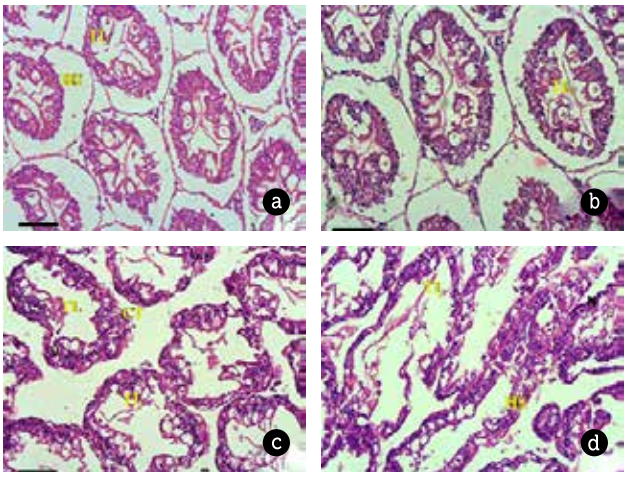

Fig. 10. (a–d) Photomicrographs of hepatopancreas of *P. homarus.* (Figs. a and b) Section of hepatopancreas collected from Kovalam

with spermatogenetic cells. The follicle lumen was filled with a large number of spindle-shaped spermatozoa. Overall, the spermatozoa are tightly packed inside the follicle lumen (Figs. 11 a and b). In contrast, the lobster collected from polluted site, showed follicle cells containing hypertrophied spermatozoa with degenerating spermatogenetic cells containing cellular debris. The free spermatozoa in the follicle lumen were stained blue, other cellular debris were stained red and showed swollen membranes suggesting that spermatozoa were dead (Fig. 11 c). In other words, the spermatozoa are arranged in a scattered manner leaving large vacuoles inside the follicle lumen. Cellular debris were also fomed between the lumen. Fibrous tissues were spread as a thin layer inside the spermatogenic cells (Fig.11 d).

Ovary: The ovary of lobster collected from Kovalam showed mature oocytes occupying about half of ovarian tissue. Follicle

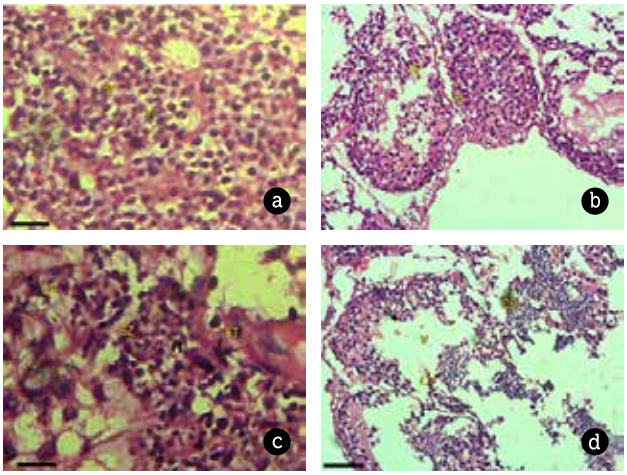

Fig. 11. (a–d) Photomicrographs of testis of *P. homarus.* Lobster collected from Kovalam coast showing normal architecture with subspherical follicles filled with spermatozoa

cells are arranged in an organized manner. (Figs.12 a and b). However, the section of ovary of lobster collected from Royapuram showed matured oocyte without yolk granules. Follicle cells showed disruption in its texture (Fig. 12 c). Degraded tissues are visible in between the follicular cells (Fig. 12 d).

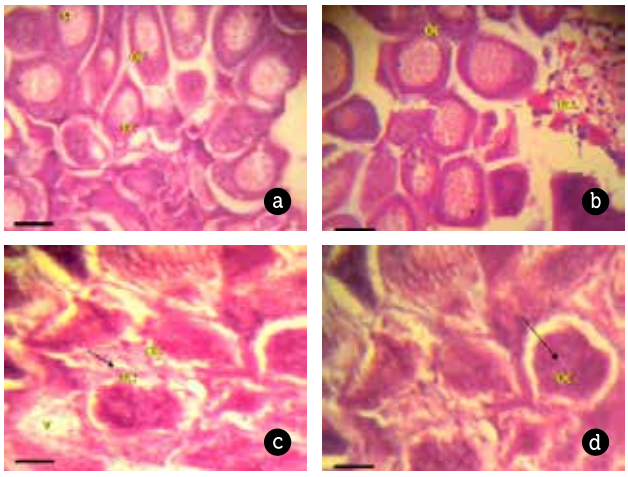

Fig. 12. (a–d) Photomicrographs of ovary of *P. homarus.* Lobsters from Kovalam showed mature oocytes occupying about half of ovarian tissue, enveloped by a row of follicle cells

Ultrastructure of gills

Scanning electron micrographs confirmed the results obtained by light microscopy. The gills in *P. homarus* collected from Kovalam coast showed normal surface topography comprising gill filaments. Filaments are joined to each other by ciliary, inter filamentar junctions. The organized lamellar epithelium signifies normal health of the tissue (Fig. 13A). In contrast, the gill of *P. homarus* collected from Ennore estuary exhibited infiltration of haemocytes from the frontal portion. Lamellar epithelium seems to be severely damaged and necrosis was observed at the tip of the filament (Fig. 13B).

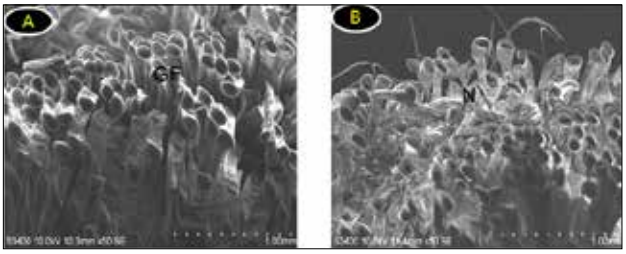

Fig. 13.(A & B) Scanning electron micrographs of the gills of *P.homarus homarus.* Lobsters collected from Kovalam shows the normal architecture of the gill filaments

Discussion

Metal bioaccumulation in aquatic organisms could pose adverse health effects to both the accumulating organisms and their higher trophic consumers, including humans (Lunden and Noren, 1998). The tissue distribution of metals can be useful for defining accumulation pathways and possible detoxification routes (Rainbow and Black, 2005; Alquezar *et al.*, 2007). In the present study, the bioaccumulation levels of different metals in various tissues indicated elevated concentrations in hepatopancreas and gills. The higher concentrations of Cu, Zn and Cd were registered in hepatopancreas from both Kovalam and Royapuram coast. Particularly high concentration of certain metals in specific organs may be related to the utilization of that metal. Notably the concentration of Zn and Cu were high in hepatopancreas, probably due to metallothioneins, which sequester these metals. Earlier studies showed an increase in copper content in the crabs collected from Royapuram coast. Meiggs (1980) found that both crabs and sediments were remarkably contaminated by copper and zinc in the vicinity of a dry dock. Hence, the use of copper in antifouling paints on boats might be the main cause of elevated levels of copper in the tissues of *P. homarus.*collected from Royapuram coast. Besides the pattern of copper accumulation in the tissues, was similar to the pattern reported by Yap *et al.* (2006). In accordance with Arockia Vasanthi *et al.* (2014) Fe concentration was found to be high in gills compared to other tissues. Fe is critically involved in enzymatic and respiratory processes of crustaceans, therefore, Fe is abundant in gills (Mohapatra *et al.*, 2009). Higher Fe content in the gills of *P. homarus* is most likely due to the presence of absorbed particulate matter on the gills rather than to the active biological uptake of the metal. High values of Fe in gill tissues had been previously reported for cancer irroratus (Martin, 1974). The concentration of cadmium obtained in the mussel was within the normal range found in mussels of other regions (Julshamn and Duinker, 2000). This concentration was clearly below the upper limit of 1.0 mg g^{-1} for mussels used for human consumption set by EU (2001). Surprisingly, the concentration of cadmium in *P. homarus homarus* encountered in this study was below the prescribed limit. Much of the cadmium accumulated by aquatic invertebrates was bound to metallothionein in the cytosol of the organ predominantly used for accumulated cadmium storage (Mason and Jenkins, 1995). In certain circumstances of severe cadmium exposure, there is indirect evidence that cadmium from metallothionein may be deposited in insoluble form in lysosomal residual bodies (Langston *et al.*, 1998). In the present study among the organs examined for the metal accumulation, minimum concentration of all the metals were observed in the muscles, several possible reasons may explain the lower accumulation of metals in muscles. The most likely reason is that muscle does not come into direct contact with the toxicant medium because it is totally covered by the skin which helps the organisms to avoid the penetration of the toxicant (Vasanthi *et al.*, 2013).

Further biochemical components can be sensitive indicators of pollutants in the surface waters. Under extreme stress conditions, proteins supply energy in metabolic pathways and biochemical reactions (Yerragi *et al.*, 2000). In the present study, the protein content decreased in all the tissues such as ovary, testis, hepatopancreas, muscle and gills of lobsters collected from the polluted site (Royapuram). Carbohydrate typically contributes to structural support and protection, and serves as nutrient and energy stores to be increased or decreased according to organisms need. In the present study, the carbohydrate content decreased significantly in all the organs of lobster collected from Royapuram.

Earlier reports explain varying degrees of histopathological changes in the gill filament (Kumaraguru *et al.*, 1982; Jiraungkoorskul *et al.*, 2002). In the present study, the section through the gills of *P. homarus homarus* collected from Royapuram coast showed irregular arrangements of gill filament with distorted cuticle and varying inter filamentary spaces. Within the secondary filament, necrosis and connective tissue damages are observed. Earlier studies have demonstrated that muscles are very sensitive to contaminants (Dyrynda *et al.*, 1997; Sauve *et al.*, 2002 and Anderson *et al.*, 1996). Muscles of *P. homarus homarus* collected from the polluted sites showed loss of muscle fiber as well as swelling of muscle fiber layer. Normal architecture of muscle bundle seems to be distorted with breakdown of muscle bundles. Similar results were observed by Maharajan *et al.* (2011) in *P. homarus homarus*.

Hepatopancreas in crustaceans is analogous to the liver in higher organisms, which is a sensitive organ and is liable to be damaged by water borne pollutants (Baticados *et al.*, 1987). The section of lobsters (collected from Kovalam) showed normal architecture of hepatopancreatic tubule and lumen. Where as, section through the lobster collected from Royapuram (Polluted site) showed lumen disruption and vacuole formation along with irregular heamal space.

Histological studies also showed abnormalities in testis and ovary in the tissues collected from polluted site, the abnormalities included follicle cells containing hypertrophied spermatozoa with degenerating spermatogenetic cells containing cellular debris, membranes suggesting that spermatozoa were dead. In other words, the spermatozoa were arranged in a scattered manner leaving large vacuoles inside the follicle lumen in testis. The section through the ovary of lobster collected from Royapuram showed mature oocytes without yolk granules and disrupted follicle cells.

Gill filaments with their large surface area are the main interface between the organism and its environment. Gills are thus continuously affected by exposure to pollutants. Hence to determine the surface topographic changes, scanning electron microscopic studies were carried on gills which revealed reduction in the number of lamellae and necrosis at the tip of gill filaments in *P. homarus* collected from Royapuram. Similar lesions have been reported in the posterior gill lamellae of various species after exposure to several metals (Bubel, 1976; Victor *et al.*, 1990 and Mazon *et al.*, 2004). However the lamella was well organized and intact in the gills of *P. homarus homarus* from Kovalam coast.

The present study thus, provides primary information on the distribution of metal concentrations in the tissues of *P. homarus homarus* from Royapuram and Kovalam coast. The differences in the pattern of metal occurrence in various organs of lobster, *P. homarus* and the significant increase in the metal concentrations are likely associated with the contributions from the surrounding industries. These results clearly indicate the ability of crustaceans to accumulate metals at detectable levels, though the levels of metals detected from both the coasts did not exceed the permissible levels, it may pose health problems for the organisms. Further studies are necessary in order to evaluate the ecological significance of these contaminants as well as monitoring programs for assessment and management purpose.

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