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Ultrastructural observations in gills and hepatopancreas of prawn *Macrobrachium malcolmsonii* exposed to mercury

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Abstract: *The juveniles of M. malcolmsonii were exposed to 24.1 µg l^{-1} of Hg for a period of 21 days. The gills and hepatopancreas of test prawns were sampled and processed for electron microscopic observations. Mitochondria are the organelle most affected in the gills of test prawns. The number of mitochondria and the electron-density of the matrix were found to be less in test prawns. The in-folding of cell membrane associated with mitochondria was absent in test prawns. This suggests that operation of the mitochondrial pumps was affected in the gills of test prawns. Vacuoles with crystalline granular inclusions were noted in the gills of test prawns. These are suggestive of metal-rich inorganic deposits or granules representing detoxified dumps of Hg. In the hepatopancreas of test prawns, the tubules exhibit vacuoles with granular inclusion and the cell cytoplasm contains electron-dense granules, which indicate a storage detoxification of Hg. The mitochondria were shrunken in the hepatopancreas of test prawns. This suggests attenuation of its function. The rough endoplasmic reticulum appeared vesiculated and dilated. These reactions denote the hyperactivity of the rough endoplasmic reticulum. Membranous whorl-like structures with myelin fibers and residual bodies were seen in the hepatopancreas of test prawns. Such structures indicate the involvement of lysosomal breakdown in detoxification process. The ultrastructural alterations are suggestive of the operation of compensatory mechanisms within the test prawns to enable it to tolerate Hg toxicity. However, these alterations would have an impact on the cellular integrity of the gills and hepatopancreas and such alterations can be taken as 'biomarkers' for assessing Hg pollution in the aquatic environment.*

Key words: *Hg, Prawn, Histopathology, Gills, Hepatopancreas*
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Introduction

Trace metals find extensive application in various industries including agricultural, domestic and pharmaceutical. Therefore, large quantities of heavy metals enter the natural environment and causing health hazards (Mishra *et al.,* 2007; Singh *et al.,* 2007; Ozdilek *et al.,* 2007; Yoon *et al.,* 2008; Singh *et al.,* 2008). Mercury(Hg) is a non biodegradable trace metals. Environmental disasters happened in Japan, Minemata disease stands as grim testament to the potential danger of mercury pollution (Tokuomi, 1968). It has highest affinity with the kidney and brain (Yoon *et al.,* 2008). Desirable properties of Hg, such as the ability to alloy with metals, liquidity at room temperature, eases of vaporizing and freezing, and electrical conductivity make this metal an industrially important; battery manufacturing, chlorine-alkali production, paints and industrial instruments are primary among its application. Therefore, there are general believes that Hg pollution verging in alarming situation. For instance, the presence of Hg in the coastal waters of Kalpakkam, Chennai, India has been reported (Selvaraj, 1999). The distribution pattern of Hg in Ennore estuary, Chennai, India was 0.0763 µg l^{-1} in water, 0.0428 µg l^{-1} in sediment and 0.013 to 0.40 µg g^{-1} in fish due to the discharge of industrial effluents (Rajathy, 1997). The distribution of mercury in seawater along the west coast of India ranged up to 0.116 g l^{-1} (Kaladharan *et al.,* 1999). Hg accumulation at 0.5 g g^{-1} wet weight in tissue of oysters sampled from a contaminated stream at Karwar, Karnataka coast, India has been reported (Krishnakumar *et al.,* 1998). Similarly in a recent report the distribution pattern of Hg in Ganga river at Varanasi, India was 0.00191 mg l^{-1} in water and 91.679 mg kg^{-1} in fish (Sinha *et al.,* 2007).

The impact of pollutants on an organism is expressed as perturbations at different levels of functional complexity. Therefore, the severity of a toxicant can be measured at the level of the molecular, cellular, tissue, organ, individual or population (Moore, 1985). The detection of responses to toxicant at the cellular or tissue level is of great value. In this angle the histopathological study of an organ reflects the impact of toxicant on metabolic processes at the cellular level (Triebskorn and Kohler, 1996; Mathur and Gupta, 2008). There are limited reports pertaining to the histopathological changes brought by heavy metals in shrimps and prawns (Couch, 1977; Nimmo *et al.,* 1977; Ghate and Mulherkar, 1979; Papathanassiou, 1983; Rao and Doughtie, 1984; Kutlu *et al.,* 2005).

The prawn, *M. malcolmsonii* occurs in abundance in the Cauvery River, South India. The culture of this species of prawn offers tremendous scope to meet the awesome challenge of providing adequate levels of nutritious food to the growing human population. Hg is the most toxic metal and is used in pesticide, biocide and herbicide formulations (Manahan, 2000). For example, the organic

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mercury compounds include aryl mercurials such as phenyl mercuric dimethyldithiocarbamate, used in paper mills as a slimcide and as a mold retardant. The alkyl-mercurials such as ethylmercuric chloride (C_2H_5HgCl) was used a seed fungicide. Such metallic pesticides are also used in pest management programmes in the agricultural lands adjacent to the Cauvery river; the run-off from these lands enters the river system. Moreover, Hg has very wider industrial and household applications. Hence, the Cauvery river systems are likely to be contaminated by Hg and other trace metals. In our earlier study, this species of prawn was found to be an accumulator of nickel (Kabila *et al.,* 1999). The heavy metals, like Hg, Cu and Ni caused several alterations in the general metabolism of *M. malcolmsonii*: these include synthesis of metallothionin, glutathione S-transferase and stress protein, HSP-70 (Kabila *et al.,* 2000a,b; Yamuna *et al.,* 2000, 2002; Bhavan *et al.,* 2008). Since Hg and other heavy metals caused several biochemical and physiological alterations in *M. malcolmsonii*, there was a necessity to understand the histopathological changes due to toxicity of these metals. Therefore, the present study was undertaken to observe the ultrastructural alterations caused by Hg in vital tissues, such as the gills and hepatopancreas of the commercially important river prawn, *M. malcolmsonii*.

Materials and Methods

Healthy juveniles freshwater prawn, *M. malcolmsonii,* were collected from the lower anicut of the river Cauvery, south India and acclimatized to laboratory conditions with ground water for two weeks. The water was renewed daily. The prawns were fed with standard pellet diet *ad libitum* both during acclimation and exposure.

The prawns used in the study were divided into two groups, each comprising 10 intermoult juveniles of *M. malcolmsonii* (total length: 4.0-5.0 cm and weight: 0.8-1.0 g). Each group was housing in an aquarium (15 l capacity). One group served as control; the other group was exposed to the sub lethal concentration of Hg, 24.1 µg l^{-1}, 1/6th of the 96 hr LC_{50} (96 hr LC_{50}: 145 µg l^{-1}). This was the highest sub lethal concentration of Hg in which mortality of the test prawns was found to be negligible. Hg solution was prepared from mercuric chloride ($HgCl_2$, analar grade, E Merck, India) in de-ionized water. The experiment was carried out for a period of 21 days, since the intermoult period of the prawn is 21±1 day under laboratory conditions and also the 21 day-chronic test offers an attractive alternative to the longer chronic test in toxicological studies (Maki, 1979). The medium was gently siphoned out daily with minimal disturbance to the prawns, and replaced by medium containing freshly prepared concentration of Hg. During the course of the experiment, the medium was not aerated. The gills and hepatopancreas were sampled from five prawns in each group on 21st day of exposure. These organs were fixed in modified Karnovsky's fixative for 16 hr. Following fixation in the primary fixative, the tissues were rinsed twice in 0.1 M phosphate buffer and post fixed in 1% osmium tetraxide for 1 hr. To facilitate infiltration with

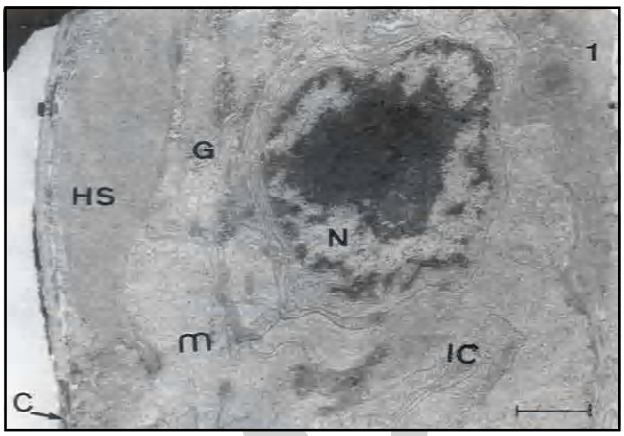

Fig. 1: Electron microphotograph of the gills of control prawns. The lamellar region of the gills showing an epithelial cell with cuticle (C), nucleus (N), hemocoelic space (HS), mitochondria (M) associated within the in-folding of cell membrane (IC) and glycogen deposits (G), x 2000 (bar = 80 µm)

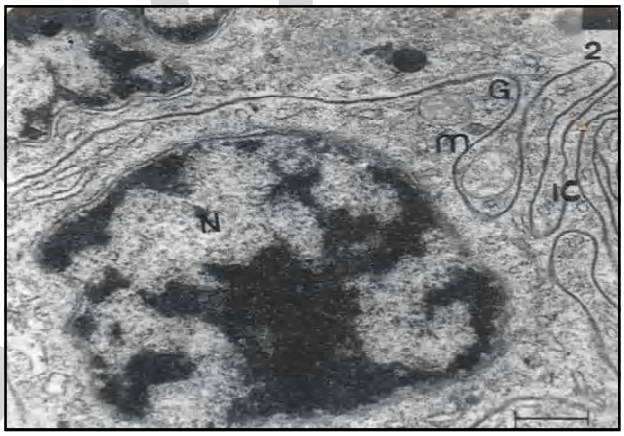

Fig. 2: Electron microphotograph of the gills of control prawns. The basal region of the gills showing cell with nucleus (N), mitochondria (M), in-folding of cell membrane (IC) and glycogen deposits (G), x 2000 (bar = 80 µm)

liquid resin, tissues were rinsed with 0.1 M phosphate buffer and dehydrated in a graded series of alcohol. The tissues were stained en-bloc in 2% uranyl acetate in 95% ethanol for an hr, cleared with propylene oxide and embedded in araldite CY 212 medium. Ultra thin sections, cut on a LKB wallac ultra microtome, were counterstained with lead citrate and uranyl acetate. All the procedures were carried out at 4°C. The image was examined under JOEL-JEM-I electron microscope.

Results and Discussion

In an organism exposed to trace metals the cellular damages occur in following states (Triebskorn and Kohler, 1996). The state of compensation (reaction), animals tolerating the adverse toxic conditions, and the state of non-compensation (loss of structural integrity), normal function of the organism is affected. In the present study, the observed ultrastructural changes in the gills and hepatoapncreas were fall in the adaptive state of compensation in *M. malcolmsonii* exposed to Hg.

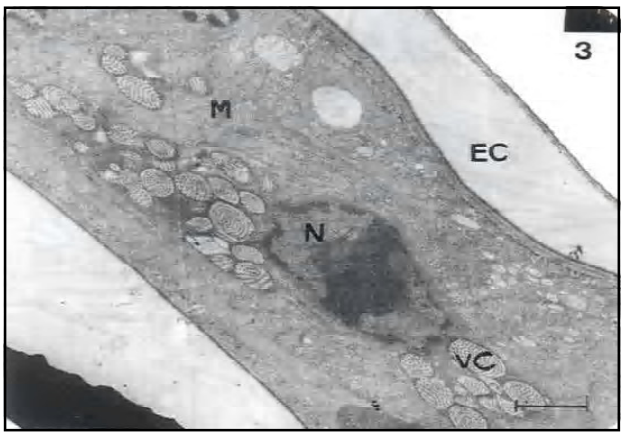

Fig. 3: Electron microphotograph of the gills of test prawns exposed to Hg. The lamellar region of the gill characterized by elevated cuticle (EC), loss of in-folding of cell membrane, presence of few mitochondria (M) and vacuoles with crystalline granular inclusion (VC), x 8000 (bar = 20 µm)

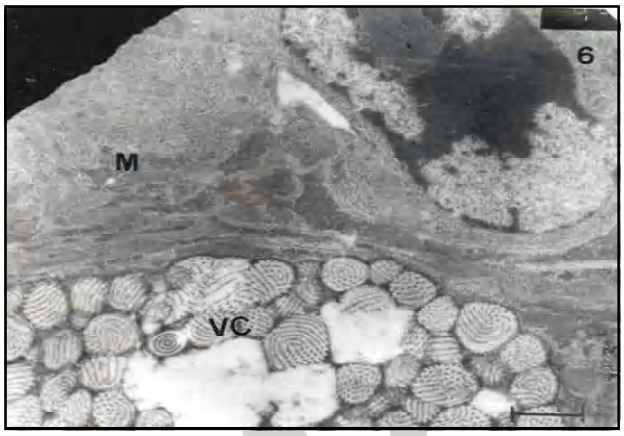

Fig. 6: Vacuoles with crystalline granular inclusions (VC) were present at the apical regions of the gills, gill cell with only few mitochondria (M) and loss of in-folding of cell membrane, x 10000 (bar = 10 µm)

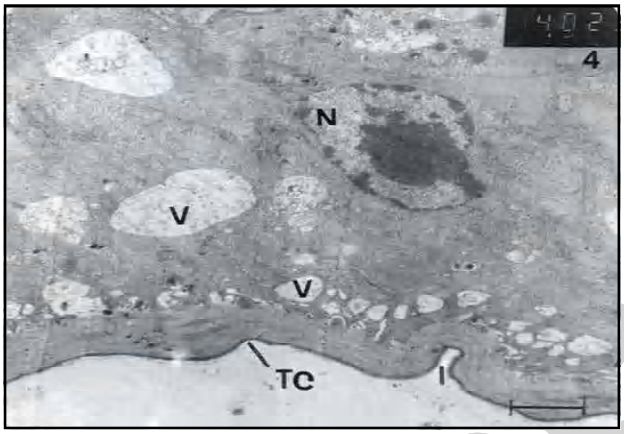

Fig. 4: The lamellar margin of the gill appeared serrated, with thickening of cuticle (TC), and the apical region of the gill had prominent invagination (I), which resulted in the formation of small vesicles (V), x 10000 (bar = 40 µm)

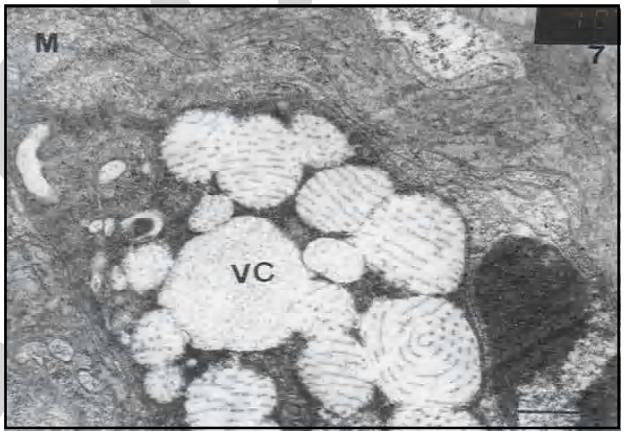

Fig. 7: Vacuoles with crystalline granular inclusions (VC) were present at the basal regions of the gills, gill cell with only few mitochondria (M) and loss of in-folding of cell membrane, x 4000 (bar = 10 µm)

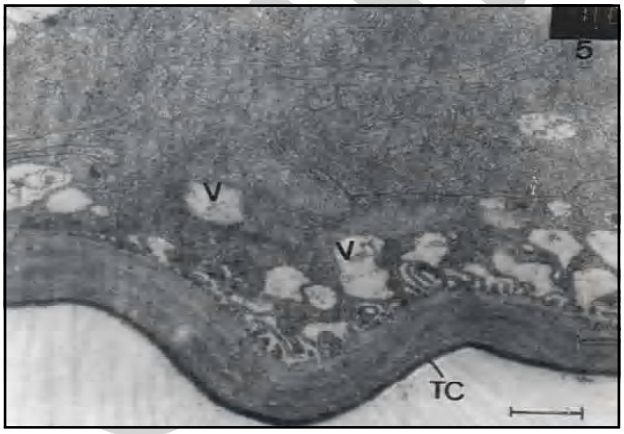

Fig. 5: The basal region of the gill also characterized by thickened cuticle (TC), with associated vesicles (V), x 8000 (bar = 20 µm)

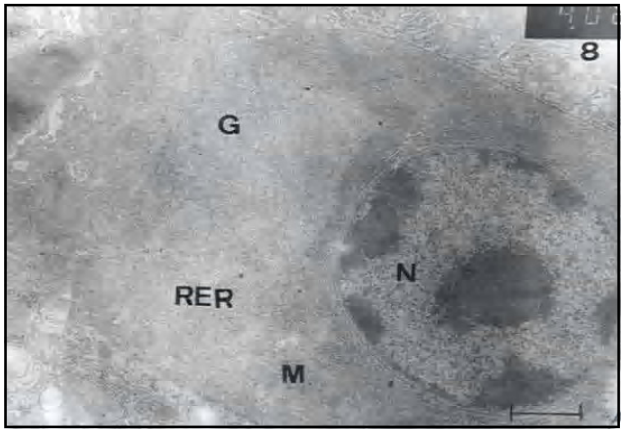

Fig. 8: Electron microphotograph of the hepatopancreas of control prawns with the embryonic (E) cell. The E-cell possess centrally placed nucleus (N), few mitochondria (M), rough endoplasmic reticulum (RER), rich in glycogen granules (G) and without microvilli, x 4000 (bar = 40 µm)

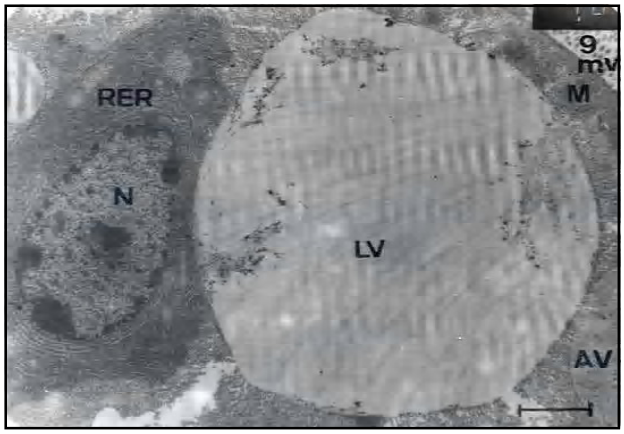

Fig. 9: Electron microphotograph of the hepatopancreas of control prawns with the absorptive (R) cell. The R-cell is characterized by a-centrically placed nucleus (N) with prominent microvilli (MV), large lipid vacuoles (LV), rich in rough endoplasmic reticulum (RER), mitochondria (M) and autophagic vacuole (AV), x 4000 (bar = 40 µm)

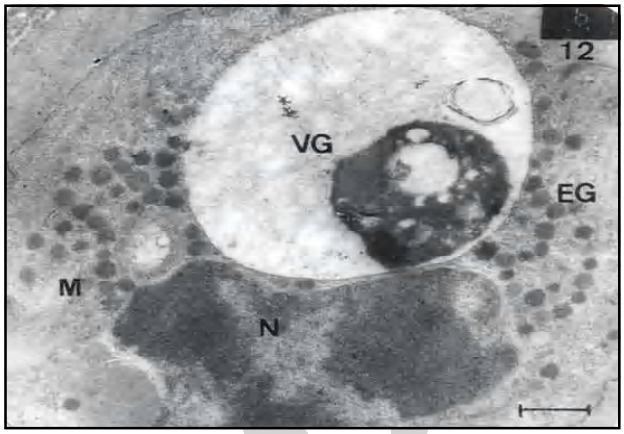

Fig. 12: Electron microphotograph of the hepatopancreas of test prawns exposed to Hg. Vacuole of hepatopancreatic cell filled with granular inclusions (VG), the cell cytoplasm contain many electron dense granules (EG) and few mitochondria (M), x 3700 (bar = 80 µm)

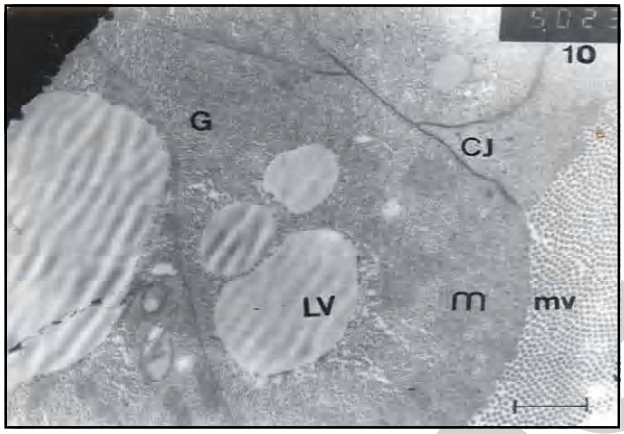

Fig. 10: Electron microphotograph of the hepatopancreas of control prawns with the secretary (B) cell. The B-cell is with lipid vacuoles (LV), microvilli (MV), few mitochondria (M), rich in glycogen (G) deposit, and clear cell junction (CJ), x 5000 (bar = 30 µm)

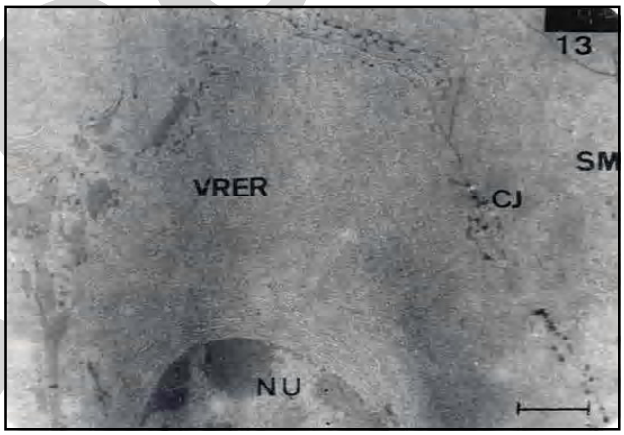

Fig. 13: Presence of vesiculated rough endoplasmic reticulum (VRER), absence of glycogen deposits, shrunken mitochondria (SM) and loss of clear cell junction (CJ) in tubule epithelium, x 8000 (bar = 30 µm)

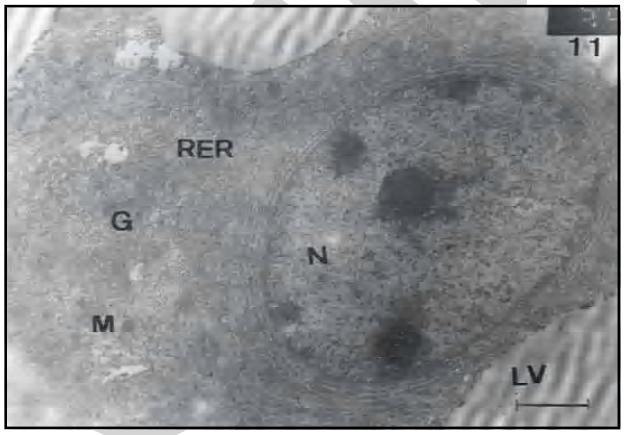

Fig. 11: Electron microphotograph of the hepatopancreas of control prawns with the fibrillar (F) cell. The F-cell is with spherical shaped nucleus (N), abundant rough endoplasmic reticulum (RER), glycogen granules (G), lipid vacuoles (LV) and few mitochondria (M), x 5000 (bar = 30 µm)

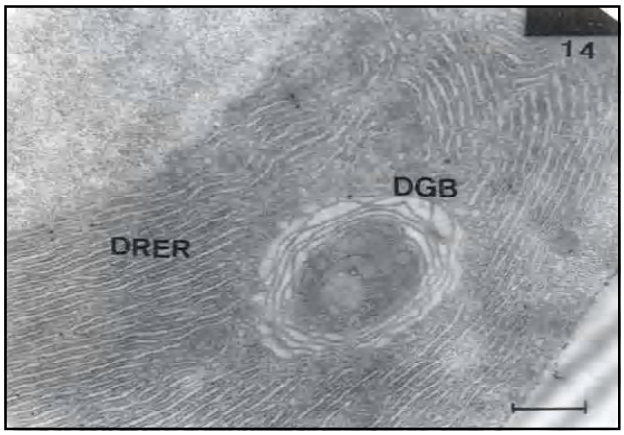

Fig. 14: Presence of dilated rough endoplasmic reticulum (DRER) and dilated cisternae of the Golgi body (DGB) in the tubule epithelium, x 4000 (bar = 40 µm)

Gills: The gills of control prawns were found to comprise an outer cuticle (C) and underlying epithelial cells and hemocoel (HS) (Fig. 1). The cuticle was found to be composed of three distinct zones; the outer and inner membranes were homogenous and electron dense, while the middle zone was electron translucent (Fig. 1). Each cell was found to possess a nucleus (N) containing dispersed chromatin and the cytoplasm was rich in glycogen (G) (Fig. 2). An abundant number of mitochondria (M) were seen to be associated/ localized with the in-folding of the cell membranes (IC) (Fig. 1, 2). Such cell in-folding are believed to play a role in the transportation of salts released by the vesicles of the cells. This is believed to be an energy-consuming process. The energy required for this process probably being derived from the mitochondria present within the in-folding. Operation of such mitochondiral pumps has been reported in the gill cells of *Crangon crangon* (Papathanassiou and King, 1983). The normal structure and integrity of the mitochondria are prerequisites to the localization and functioning of the enzymes with which they are associated. Generally, mitochondria with closely packed cristae exhibit high metabolic activity. In the present study, the mitochondria (M) in the gills of control prawns (Fig. 1,2) exhibited such structural appearances and were therefore believed to possess high metabolic activity.

In the gills of test prawns exposed to Hg, following structural alterations were observed. The outer layer of the cuticle was found to be elevated (EC) (Fig. 3). The lamellar margin appeared serrated, with thickening of the epithelial lining (TC) (Fig. 4). The apical regions of the cell had prominent invaginations (I) which in turn resulted in the formation of small vesicles (V) (Fig. 4). The basal region of the gills also showed a thickened cuticle (TC) with associated vesicles (V) (Fig. 5). Similar observations have also been reported in shrimps and prawns exposed to heavy metals (Couch, 1977; Nimmo *et al.,* 1977; Ghate and Mulherka, 1979).

Mitochondria (M) are the organelle most affected in the gills of test prawns. The number of mitochondria and the electron-density of the matrix were found to be less in the gills of test prawns than that of control prawns (Fig. 3-7). Further, in-folding of cell membrane (IC) associated with mitochondria was absent in test prawns (Fig. 3-7). This suggests that operation of the mitochondrial pumps was affected, which in turn could have reduced the uptake of available ions. The changes noted in the mitochondria might have lead to low activity of the Kreb's cycle enzymes and the consequent loss in the aerobic production of ATP and thus, suggests a probable shift towards an anaerobic respiration as has been reported in the gills of *Jeara nordmanni* exposed to Cu, Hg and Cd (Bubel, 1976).

In our earlier study, the concentration of metallothionein was found to be significantly elevated in the gills and hepatopancreas of *M. malcolmsonii* exposed to Ni (Kabila *et al.,* 2000a). Vacuoles with crystalline granular inclusions (VC) were noted in the gills of test prawns (Fig. 3, 6 and 7). These are suggestive of metal-rich inorganic deposits or granules representing detoxified dumps of toxic metals. The absence of myelin structures in association with the Golgi apparatus and lysosomal bodies (Fig. 3-7) suggest that the

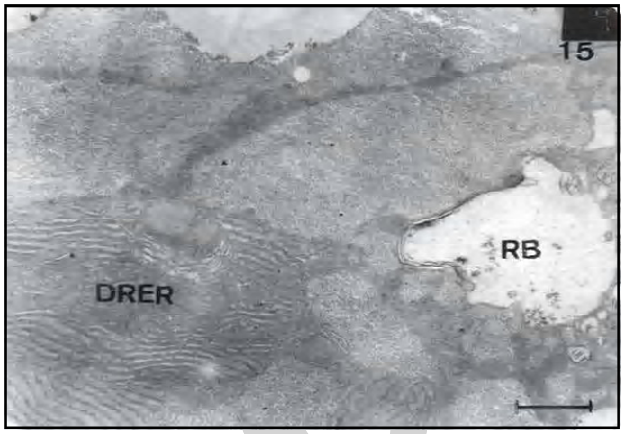

Fig. 15: Presence of residual body (RB) in the cell cytoplasm of the hepatopancreas, x 4000 (bar = 40 µm)

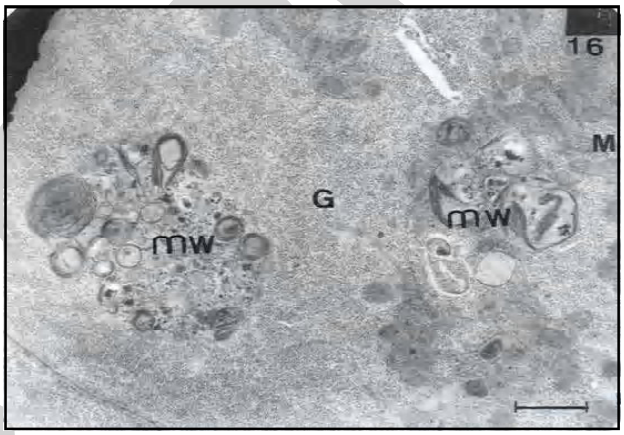

Fig. 16: Presence of membranous whorl like structure (MW) with myelin figures at ends of the tubule epithelial cell and accumulation of mitochondria (M) around the membranous whorl, x 6700 (bar = 30 µm)

breakdown products of metal-binding protein (metallothionein) were en-routed through the pyrophosphate pathway, and this represents the detoxified dump of toxic metals (Brown, 1982; Nott and Langston, 1989; Pullen and Rainbow, 1991). Metal rich inorganic deposits or granules have also been reported in marine invertebrates (Simkiss *et al.,* 1982; Taylor and Simkiss, 1984; George, 1990).

Hepatopancreas: In crustaceans the hepatopancreas have several functions, including the secretion of digestive enzymes, the absorption of food and the storage of lipid material, glycogen, and minerals. The hepatopancreas of *M. malcolmsonii* comprises numerous blind-ending tubules, bound together by connective tissue and four types of cells could be differentiated in the tubules of control prawns (Fig. 8-11). The embryonic (E) cells were characterized by a centrally placed nucleus (N), proportionately large when compared to the volume of the cell. Microvilli were absent. The cytoplasm possessed few mitochondria (M) and rough endoplasmic reticulum (RER) and was rich in glycogen (G) (Fig. 8). The absorptive (R) cells were characterized by lipid vacuoles (LV). The nucleus (N) was a-centrically placed, and was surrounded by the rough endoplasmic reticulum (RER) and mitochondria (M). Prominent

microvilli (MV) and autophagic vacuoles (AV) were also present in this cell type (Fig. 9). The secretory (B) cells were characterized by a large number of lipid vacuoles (LV). The apical membrane had an undulated appearance and the microvilli (MV) were more pronounced. A smaller number of mitochondria (M) were seen. The cells had clear cell junction (CJ) (Fig. 10). The fibrillar (F) cells had abundant rough endoplasmic reticulum (RER) with flocculent material, predominantly around the nucleus. These cells had few mitochondria (M). Lipid vacuoles (LV) and glycogen (G) granules were present (Fig. 11).

The crustacean hepatopancreas is the principal site for the accumulation and detoxification of metals (Loizzi, 1971). In our earlier study, synthesis of metal binding protein has been observed in *M. malcolmsonii* on exposure to Ni (Kabila *et al.,* 2000a). Several changes were observed in the cell organelles of the hepatopancreas of the prawns exposed to Hg. Nucleus (N) had dispersed chromatin. The cytoplasm was non-homogenous, the tubules exhibited vacuoles with granular inclusion (VG) and the cell cytoplasm contains electron-dense granules (EG) (Fig. 12). Such granules suggest that the Hg bound to protein is precipitated in the form of granules, which indicates a storage detoxification of Hg in test prawns. The occurrence of such deposits (copper rich granules) has been reported in the barnacle, *Semibalanus balanoides* and believed that they were derived from metallothionein breakdown (Walker, 1977). Such structures have also been reported in marine animals due to metal toxicity (George, 1990).

The mitochondria are sensitive to many kinds of stressors and react very quickly with general pathological symptoms such as swelling, shrinkage, disruption of cristae or formation of intra-mitochondrial crystals (Triebskorn, 1989). In the hepatopancreas of test prawns, the cytoplasm appeared as non-homogenous with shrunken mitochondria (SM), and clear cell junction (CJ) was lost (Fig. 13). The shrinkage noted in the mitochondria suggests that its function had been attenuated. The endoplasmic reticulum also responds very quickly in the hepatopancreas of test prawns, the rough endoplasmic reticulum appeared vesiculated (VRER) (Fig. 13) and dilated (DRER) (Fig. 14). These reactions denote the hyperactivity of the rough endoplasmic reticulum, and suggest enhanced detoxification of Hg. Similar suggestions have been derived in slug against molluscicides and heavy metal toxicity (Triebskorn and Kohler, 1996). The cisternae of the Golgi body (DGB) were also appeared dilated (Fig. 14) in the hepatopancreas of test prawns. The route of detoxification leads to the production of metal-rich deposits or granules by possessing membranous whorls (MW) with myelin figures and residual bodies (RB) in the cell cytoplasm of the hepatopancreas (Fig. 15 and 16) of test prawns. This indicates the fact that lysosomal breakdown of metallothionein was possibly operative in test prawns. Presence of dilated membranous structure and myelin bodies has also been reported in the shrimp, *Palaemonetes turcorum* exposed to lead acetate (Kutlu *et al.*, 2005).

The ability of an organism to survive in contaminated environment is often correlated with various mechanisms of detoxification including the precipitation of metals as different types of intracellular granules. In the present study, one such type of granule formation, vacuoles with crystalline granular inclusions (VC) in the gills noted suggests the binding of Hg to phosphate, predominantly in the form of pyrophosphate. Moreover, the vacuoles filled with granules (VG) and the electron-dense granules (EG) noted in the hepatopancreas indicate storage detoxification of Hg in test prawns. The lysosomal breakdown of metallothionein was also operational in the hepatopancreas of test prawns exposed to Hg. This was evident from the presence of membranous whorls (MW) with myelin figures and residual bodies (RB). Operations of such detoxification mechanisms imply the ability of *M. malcolmsonii* in neutralizing Hg toxicity.

In conclusion, the structural alterations seen in cell organelles, such as dilation or vesiculation of rough endoplasmic reticulum, increase or decreases of the electron-density of the mitochondrial matrix and swelling of the mitochondria following exposure to Hg, suggest functional alterations of the organelles and not a pathogenic response (Nott and Moore, 1987; Triebskorn and Kohler, 1996). Therefore, such changes noted in the present study can be taken towards operation of compensatory mechanism against Hg toxicity. The operation of such mechanism achieved by detoxification of Hg prevents severe cellular damage. However, the ultrastructural changes recorded in the gills and hepatopancreas of test prawns would have an impact on the cellular processes of these two vital organs. Therefore, such changes can be taken as 'biomarkers' for screening Hg pollution in aquatic environment.

Acknowledgments

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