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Research Article

EFFECTS OF COPPER ON THE ULTRASTRUCTURAL ALTERATIONS IN THE GILLS OF ESTUARINE HERMIT CRAB, *CLIBANARIUS INFRASPINATUS*

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ABSTRACT

To study the histological changes of the gill due to copper in the estuarine hermit crab *Clibanarius infraspinatus* in effects of chronic exposure for 10, 20 and 30 days were examined under the light, scanning and transmission electron microscopy. The experimental setup was made the 30 x 30 x 50 cm at temperature 28±1 ºC, pH 7.9±0.2, 27±1 ppt salinity and 5.6±0.2 mg/l dissolved oxygen. The LC_{50} of copper 5, 6, 7, 8, 9 and 10mg/L concentration level added with 5L of water. The mortality was 24, 48, 72 and 96 h and the LC_{50} $1/10^{th}$ for 96 h was taken as the SL study for 30 days of exposures hermit crab selected following this histological studies. In Light Microscope at 58-60°C, the SEM and TEM. The effects of chronic exposure for 10, 20 and 30 days to 10% sub lethal(SL) concentration of copper 96 hr LC_{50} - 8.547 mg/l; 10% SLC 0.8547 mg/L). The present ultra structural alterations were remarkable during the exposure period revealed changes observer serve as "biomarkers" for assessing heavy metal toxicity in aquatic environment.

Keywords: Hermit crab, *Clibanarius infraspinatus*, Copper, Gills, SEM, TEM.

INTRODUCTION

In recent days water pollution has been increasingly at an alarming rate due to urbanization, modernization, industrialization and agricultural runoff. Most of these pollutants especially heavy metals and pesticides are strongly absorbed in the soil and sediments can persist for a long periods without degradation. Among the industrial wastes, heavy metals cause a hazard not only to mankind but also to other plants and animals. Heavy metals are non – degradable and are regarded as hazardous to the aquatic system for their environmental persistence and their ability for accumulation (Sibel and Altindag, 2006). The heavy metal copper is essential however high concentration of these metals may become toxic at the concentrations which exceed the required limits (Wright and Welbourn, 2002). The copper a component of respiratory pigment haemocyanin is in the crustacean species (Bryan, 1968). They are essential for normal physiology of crustaceans. However, they become toxic if high environmental concentrations are attained.

The impact of pollutants on an organism is expressed as perturbations at different levels of functional complexity. Therefore, the detection of responses to toxicant at in tissue level is of great value (Mathur and Gupta, 2008). In this angle the histopathological study are capable of detecting the slightest cellular damage resulting from the exposure to toxic polluting agents (Kohler *et al*., 2002).

Several experimental studies have also been conducted on the crustaceans exposed to variety of metallic toxicants to achieve the purpose of studying the histopathology of the organs such as gills. The toxic effects of heavy metals mercury, copper and zinc on the prawn, *Metapenaeus dobsoni* (Sivadasan *et al*., 1986). The effect of heavy metal copper on the gill epithelium of *Carcinus maenas* (Lawson *et al.*, 1995). The studies were conducted on the effect of

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mercury on the gill ultrastructure of prawn, *Macrobrachium malcolmsonii* (Yamuna *et al.,* 2009).

Hermit crabs are scavengers and help quickly recycle the dead matter on the shores. They are regarded as appropriate bio-indicator for metal concentration in estuarine ecosystem and it can be used effectively in toxicity studies (Ajmal Khan *et al.*, 1986; Lyla and Ajmal Khan, 2011). Information about toxicity studies in these organisms are a few. Hence an attempt has been made to study the histological changes of the gill due heavy metal copper in the estuarine hermit crab *Clibanarius infraspinatus.*

MATERIALS AND METHODS

Study area

The hermit crab, *C.infraspinatus* (carapace length 15-20mm) were selected for the experiment and were collected from Agniar estuary (Lat.10º 20'N; Long 7º9 23'E) is situated in the Palk Strait on the Pudukottai district east coast of India. Agniyar basin is the main source of surface water and river basin consists of three sub basins, namely Agniyar , Ambuliyar and south vellar. There are seven tributaries in ths basin.

Test chemical of the analytical grade copper sulphate ($CuSo_4.\ 5H_2O$) was obtained from New India Chemical Enterprises, Cochin, India and used without further purification for the experiment.

Animal maintenance

The collected samples were safely brought to the laboratory and acclimatized for one week in the fiberglass tank with dimension (30X30X50cm).The water was renewed daily to avoid accumulation and contamination of excretory materials and feeding was withheld 24 h before the commencement of the experiment. In the present study estuarine water was used which had the following physico – chemical characteristics: temperature 28±1 ºC, pH 7.9±0.2, salinity 27±1ppt and dissolved oxygen 5.6±0.2 mg / l.

Preparation of stock solution and determination of 96 h LC_{50} value of copper

Stock solution of copper was prepared by dissolving 1g of copper in an appropriate amount of water. For the determination of median tolerance limits or LC_{50} different concentrations of cadmium (5, 6,7,8,9 and 10mg/L) were prepared from the stock and added in separate fiberglass tank containing 5L of water. Three replicates were maintained for each concentration and 10 hermit crabs of similar size were introduced. The test water was renewed at the end 24h and freshly prepared copper was added to maintain the concentration of copper at a constant level. A concurrent control of 10 hermit crabs in three different fiberglass tanks was maintained under identical conditions. The mortality was recorded after 24, 48, 72 and 96 h, and median lethal concentration (LC_{50}) values were calculated by the Finney method (Finney, 1964). $1/10^{th}$ value of the LC_{50} value for 96 h was taken as the sublethal concentration (Sprague, 1971).

Sublethal studies

For sublethal toxicity tests 40 hermit crabs were selected and divided into four groups (one control and three experimental) with 10 hermit crabs in each fiberglass tank filled with water. The desired concentration (1/10 of 96h LC_{50}) of the toxicant was added directly in order to maintain constant concentration of the toxicant. The experiment was conducted for 30 days and sampled at 10 days interval and no mortality was observed during the above treatment period. At the end of the stipulated periods (10^{th}, 20^{th} and 30^{th} day) of exposures hermit crab were randomly selected and sacrificed for histological studies.

Light Microscopic Studies

On 10, 20 and 30^{th} day hermit crab were taken out, sacrificed and the gill was excised out. The gill tissue was fixed in Bouin's fluid and then they were processed (Sibel and Altindag, 2006) and embedded in paraffin wax (58 – 60°C). Serial sections of 8 µm thickness were cut and deparafinshed sections were stained in haematoxylin and counterstained with aqueous eosin (Gurr, 1962).

SEM studies

The gills were dissected, washed in 1% phosphate buffer and fixed in 3% glutaraldehyde. Then gill tissues were dehydrated in a graded alcohol – acetone mixture series dried using the critical point technique. The dried gills were mounted on the stub and were coated with gold in a gold coating unit. The morphology of the gills was examined under a JOEL JSM 6360 SEM Japan (Roy and Munshi, 1991).

TEM study

The gill of hermitcrabs for TEM study was fixed in 3% gluataraldehyde and washed in sodium cacodylate – sucrose buffer followed by post fixation in 1% osmium feroxide solution. These were dehydrate by ascending seroies graded alcohol and cleaning by propylene oxide. After degradation the tissue was infiltered by propylene

oxide and epoxylesin and then embedded in siligonised rubber mould with exposed resin

Ultration section was cut through ultra microtome wit diamond knife. Ultra thin section was taken on copper grid and stained with uranylacetate and Reynold's solution and viewed in EM Philips 201C and photographed (Reynolds, 1963).

RESULTS

LC_{50} value of Copper

The results of the acute toxicity test are presented in Table 1. Percent mortality of *Clibanarius infraspinatus* exposed to different concentration of the heavy metal copper revealed LC_{50} values of 8.89, 8.73, 8.63 and 8.54 ppm for 24, 48, 72 and 96 hrs exposure periods respectively (Figure 1-4). Analysis of extent of scatter of observed LC_{50} values of copper indicated a lower confidence limit (LcL) of 8.97 and upper confidence limit of 8.80 for 24hr.

Light microscopic observation

Histology of Control gill: In control hermit crabs the gill filament showed uniform arrangement of lamellae (GL) with interlamellar space (ILS). The normal haemocoelic spaces with haemocytes were observed (Figure 5).

Histological changes of Cu treated gill: In sublethal exposure of Cu, the gill of *Clibanarius infraspinatus* showed marked histological changes. Appreciable changes were noticed in the histology of gill after 10 days treatment including fusion of lamellae, swelling of gill lamellae and accumulation haemocytes (Figure 6)

The damage was more severe and progressive after 20 days of exposure. The gill lamellae were damaged to a great extent. Disintegration of epithelium, abnormal gill tips and lamellar fusion were observed (Figure). However such changes were drastic to the extent that necrosis, fusion gill lamellae and hyperplasic lamellar epithelium were found in the 30 days treated hermit crabs (Figure 8).

SEM observation

Control gills: In gills of control *Clibanarius infraspinatus*, the gill lamellae appeared mucous free with normal architecture (Figure 9).

Alterations of gills exposed to copper: The damages, fusion of secondary lamellae were observed after 10 days of exposure (Figure 10). On exposure to Cu for 20 days, necrosis and degeneration of gill lamellae were observed (Figure 11). In hermit crab, treated up to 30 days, the changes observed in the gill of *Clibanarius infraspinatus* were degeneration of gill lamellae with mucous opening (Figure 12).

TEM observation

Ultra structure of control gills: Transmission electron microscopy of gill from the control group showed distinct cell junction, a round nucleus with a prominent central nucleolus (Figure 13). The cytoplasm contains mitochondria (M), rough ER and Golgi bodies.

Ultra structural alterations of gills exposed to copper: In hermit crab at 10 days of Cu treatment, swollen of rough endoplasmic reticulum, and loss of cristae in mitochondria were noticed (Figure 14). At 20 days of exposure, pyknotic nucleus and thickening of cuticle were also observed (Figure 15). After 30 days of treatment, ultra structural alterations in the gills, such as pyknotic nucleus, loss of cristae in mitochondria and degranulation of rough endoplasmic reticulum were most prominent (Figure 16).

Table 1. Percent mortality of *C. infraspinatus* exposed to different concentrations of copper for different periods (24-96h).

Hours of Exposure	LC_{50}	L.C.L	U.C.L	Regression Equation	Calculated χ^2 value	Table χ^2 value
24	8.890679	8.979161	8.80307	Y= -31.88532+38.87023 X	2.77048	11.07
48	8.738555	8.839954	8.638319	Y= -38.05661+45.73486 X	11.48737	11.07
72	8.630301	8.666496	8.594256	Y= -45.19206+53.62251 X	6.85611	12.59
96	8.547151	8.588732	8.505767	Y= -38.89946+47.11146 X	6.85244	11.07

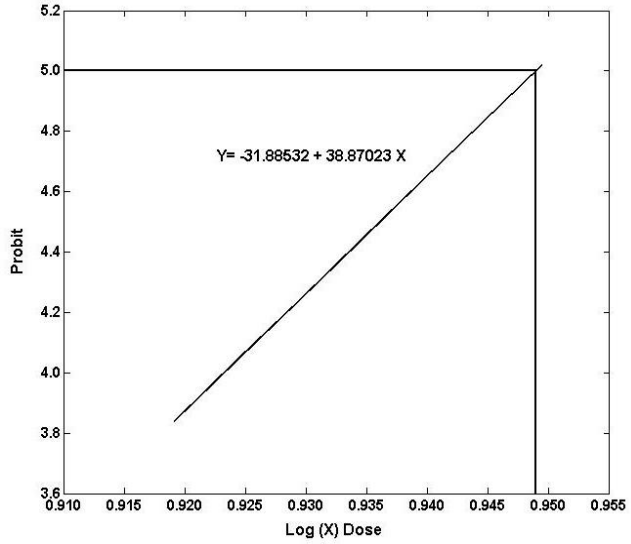
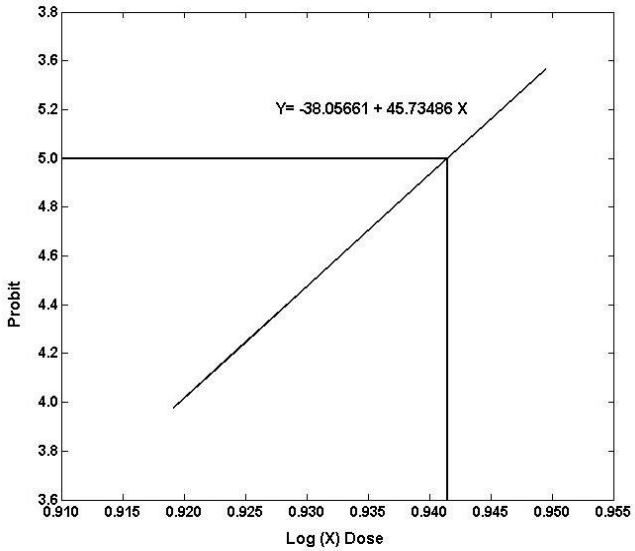


Figure 1



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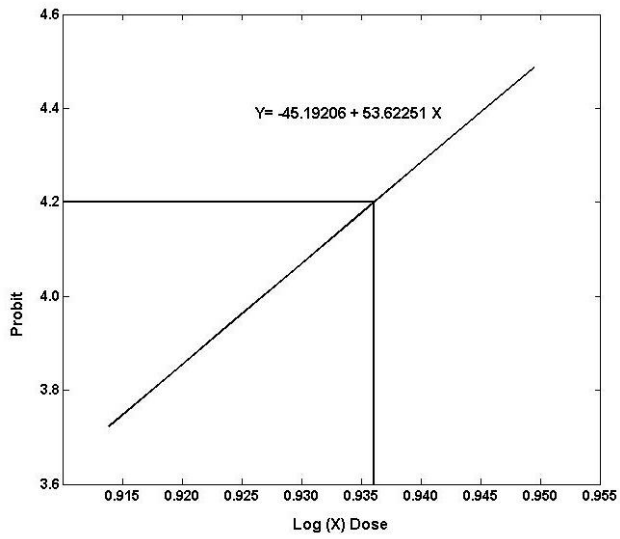

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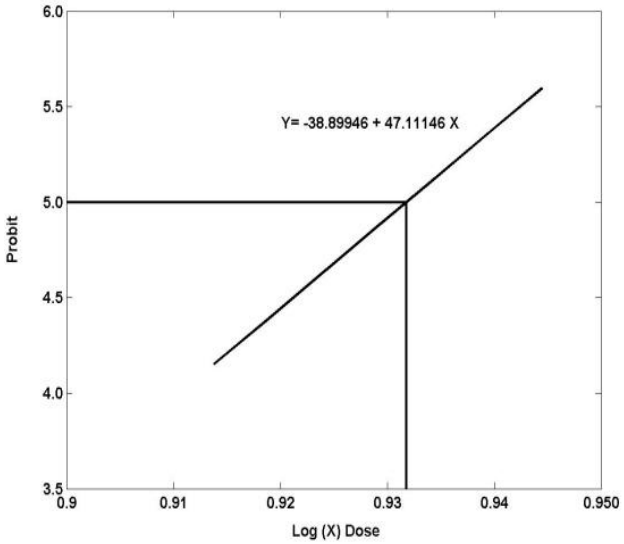


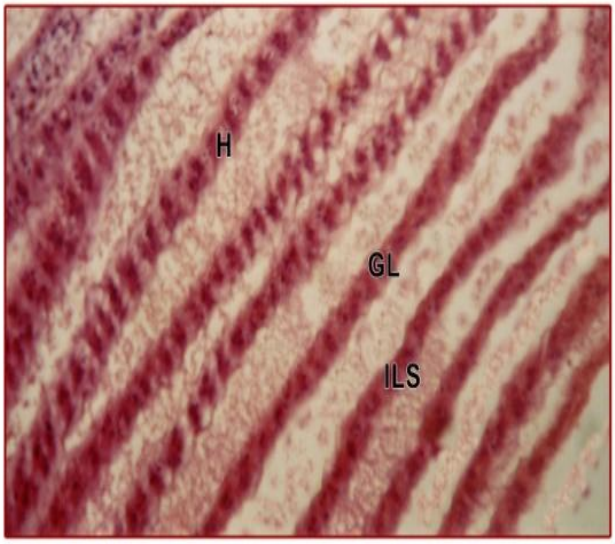
Figure 4

Figure 1. Regression line (based on profit analysis) of long concentration of copper vs percent mortality of hermit crab for 24 hours.

Figure 2. Regression line (based on profit analysis) of long concentration of copper vs percent mortality of hermit crab for 48 hours.

Figure 3. Regression line (based on profit analysis) of long concentration of copper vs percent mortality of hermit crab for 72 hours.

Figure 4. Regression line (based on profit analysis) of long concentration of copper vs percent mortality of hermit crab for 96hours.



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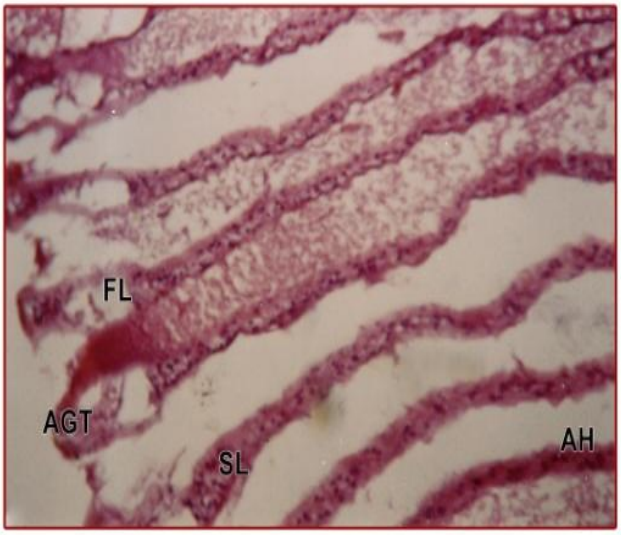

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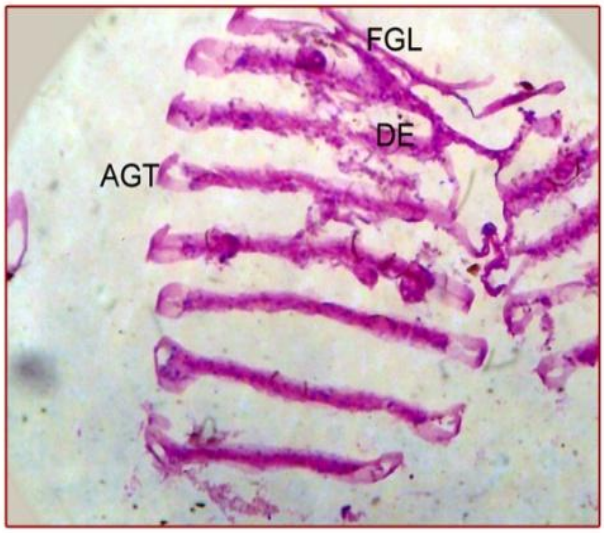

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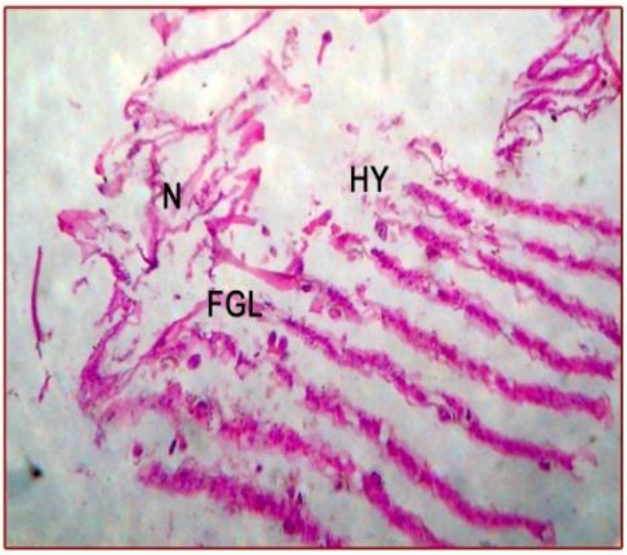


Figure 8

Figure 5. Light micrographs of control hermit crab gill lamellae H – Haemocytes, GL - Gill Lamella, ILS - Inter Lamellar Space.

Figure 6. Gill of 10 days Cu treated hermit crab FL-Fusion of Lamellae, AGT-Abnormal Gill tips SL-Swelling of Gill Lamellae, AH-Accumulation of Haemocytes.

Figure 7.Gill of 20 days Cu treated hermit crab FGL - Fusion of Gill Lamellae, DE - Disintegration of Epithelium, AGT - Abnormal Gill Tips.

Figure 8. Gill of 30 days Cu treated hermit crab N – Necrosis, FGL - Fusion of Gill Lamellae, HY – Hyperplasia.

Figure 9

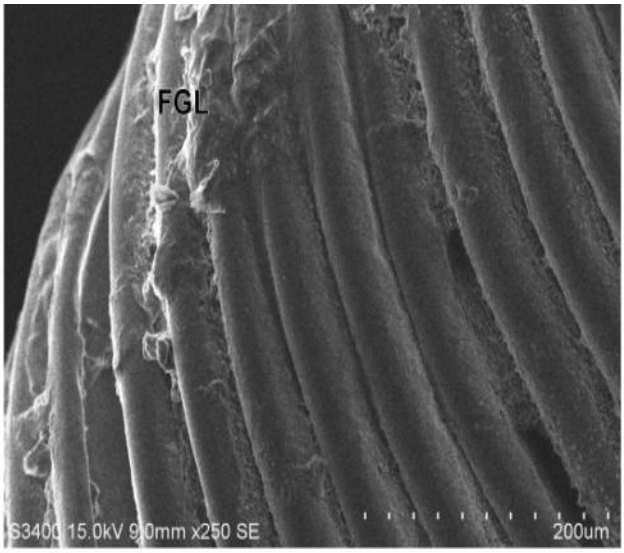
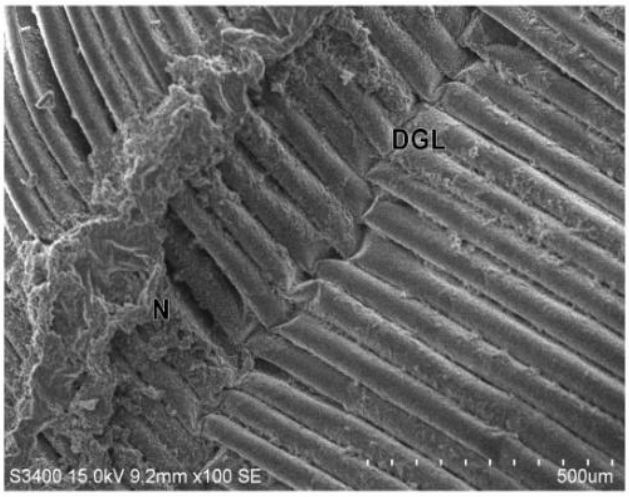


Figure 10



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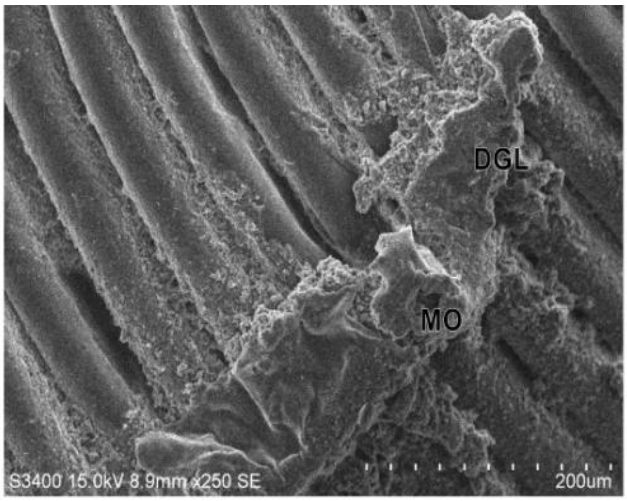


Figure 12

Figure 9. Scanning electron micrographs of the gill of control hermit crab Gill Lamellae (GL).

Figure 10. Fusion of gill lamellae (FGL) of the gill of 10 days Cu treated hermit crab.

Figure 11. Degeneration of gill lamellae (DGL), necrosis (N) of the gill of 20 days Cu treated hermit crab.

Figure 12. Degeneration of gill lamellae (DGL), mucous opening (MO) of the gill of 30 days Cu treated hermit crab.

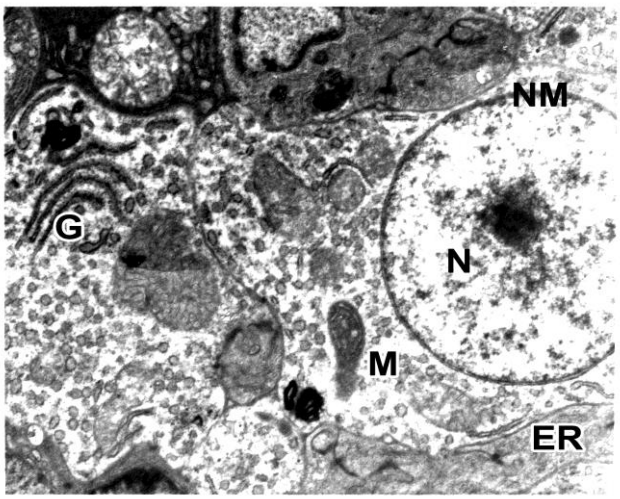
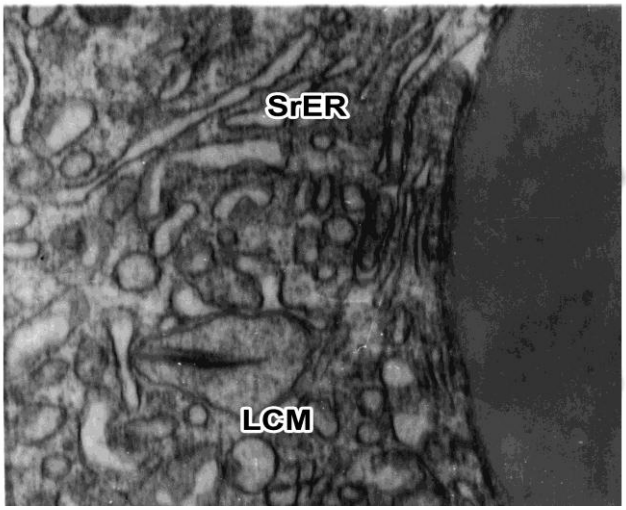


Figure 13



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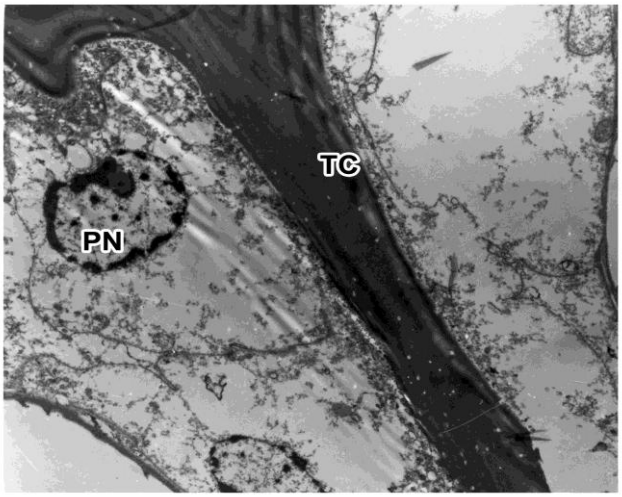

Figure 15

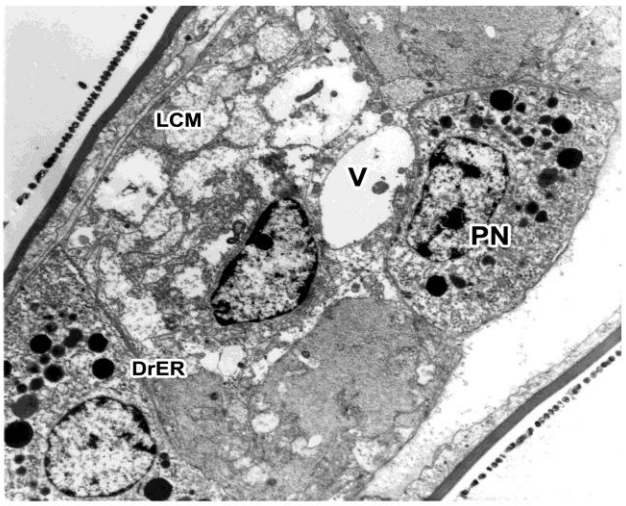

Figure 16

Figure 13. Transmission electron microphotograph of the gills of control hermit crab N-Nucleus, NM-Nuclear Membrane, ER-Endoplasmic Reticulum, G-Golgi Complex.

Figure 14. Transmission electron microphotograph of the gills of 10 days Cu treated hermit crab SrER-Swollen of Rough Endoplasmic Reticulum, LCM-Loss of Cristae in Mitochondria.

Figure 15. Transmission electron microphotograph of the gills of 20 days Cu treated hermit crab PN-Pyknotic Nucleus, TC-Thickening of Cuticle.

Figure 16. Transmission electron microphotograph of the gills of 30 days Cu treated hermit crab PN-Pyknotic Nucleus, V-Vacuoles, LCM-Loss of Cristae in Mitochondria, DrER-Degranulation of Rough Endoplasmic Reticulum.

DISCUSSION

In the hermit crab, *Clibanarius infraspinatus*, 96 hrs LC_{50} value of copper was 8.547 ppm. Our findings were closely related to Lyla and Ajmalkhan (2011), whose value was 8 ppm in *Clibanarius longitarsus*. Nagabushanam *et al.* (1986) reported in *Scylla serrata* that was 34.6 ppm 96 hrs. The LC_{50} value which is nearly four times greater than our value. Senthil kumar *et al.* (2007) stated that the 96 hrs LC50 value of the crab, *Spiralothelphusa hydrodroma* was 254.68 ppm, which is much higher than our values. Valarmathi and Azariah (2003) reported that the crab, *Sesarma quadratum* showed 28 ppm, which is much greater than our value, which may be due to size variations and high degree of resistance to heavy metals.

In aquatic organisms, the gills are regarded as a major site of respiration, osmoregulation and excretion and remain in close contact with the external environment and particularly sensitive to changes in the quality of water and considered the primary target of the contaminants. For this reason, they are considered excellent indicators of environmental quality (Wendelaar Bonga, 1997).

In hermit crabs, histological lesions induced by heavy metal copper in the gill tissue were characterized by swelling and fusion of gill lamellae, abnormal tips and necrosis. Saravana Bhavan and Geraldine (Saravana and Geraldine, 2000; Saravana and Geraldine, 2009) noted similar types of gill lesions in endosulfan and carbaryl treated *Macrobrachium malcolsomi*. Lawson *et al.* (1995) and Hebal *et al.* (1999) observed epithelial hyperplasia and necrosis in copper treated shore crab *Carcinus maenas*. Ghate and Mulherkar (1979) also noted severe gill lesions in copper sulphate treated freshwater prawn *Macrobrachium kistensis*. In the present study, necrosis and fusion of gill lamellae were apparent in *C. infraspinatus* exposed to copper. These observations are quite comparable to pathological lesions induced in gills by hexavalent chromium in *Scylla serrata* (Nikanth and Savant, 1993) by Hg, Cd, Pb, As and Se treatment in *S. serrata* (Krishnaja *et al.*, 1987) and by treatment with nickel in field crab *Paratelphusa hydrodromous* (Abraham and Radhakrishnan, 2002). Victor *et al.* (1994) also noted accumulation of haemocytes in gills and necrosis in the gill tissues of lead chloride treated *P. hydrodromous*. In the present study, the gill lamellar epithelium of heavy metal treated hermit crabs was completely desquamated and lamellae become shapeless at 30 days of exposure. These observations were agreement with the results reported by Couch (1977) in *Penaeus duorarum* treated with cadmium.

The TEM findings, in the present investigation on the ultrastructure of gill of hermit crabs exposed to copper was very similar to those of Papathannassiou and King (1983) on the gills of *Palaemen elegans* and Papathannassiou (1985) on the gills of brown shrimp *Crangon crangon*. Most ultrastructural changes such as swollen mitochondria, loss of cristae have already been reported in the gills of marine shrimp exposed to cadmium (Couch, 1977) and in *Macrorachium malcolmsonii* gills exposed to mercury (Yamuna *et al.*, 2009). The ultrasturctural alteration of SEM observed in gills of hermit crabs exposed to Cu comprised reduction in the number of ER cisternae and dilation of cisternae.

CONCLUSION

In the present study, it can be stated that copper exposure during sublethal treatment produces severe toxic effects on the respiratory organ of the estuarine hermit crab, *Clibanarius infraspinatus*. The finding of the present study indicate that ultrastructural changes observer serve as "biomarkers" for assessing heavy metal toxicity in aquatic environment.

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