

# Bioaccumulation and ultrastructural alterations of gill and liver in Asian sea bass, *Lates calcarifer* (Bloch) in sublethal copper exposure

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**Abstract** – Bioaccumulation and ultrastructural alterations in gill and liver tissues of 3-month old Asian sea bass, *Lates calcarifer*, in response to copper were studied by transmission electron microscopy (TEM). Fishes were exposed to two sublethal concentrations of copper (6.83 ppm and 13.66 ppm) for a period of 28 days. Accumulation of copper were higher in the liver followed by gills. The damaging effects of the histoarchitecture of gill tissues in copper-exposed sea bass were hypertrophy and hyperplasia of epithelial cells, severe oedema, telangiectasia and secondary lamellar fusion. In addition extensive vasodilation with stretching and necrosis of pillar cells were also noted. The liver showed hydropic swelling of hepatocytes with nuclear pyknosis and chromatin condensation. Blood congestion in sinusoids and accumulations of lipid droplets in the hepatocytes at higher concentrations were observed.

**Keywords:** Bioaccumulation / Ultrastructure / Gills / Liver / *Lates calcarifer*

## 1 Introduction

Application of environmental toxicological studies on non-mammalian vertebrates is rapidly expanding. For aquatic systems, fishes have become valuable indicators of the effects of noxious compounds (Khidr and Mekawy 2008). Heavy metals represent one of the major environmental problems causing long term effects on marine ecosystems. Similar to some organic contaminants, inorganic elements reaching directly or indirectly the coastal water, are provided mainly from increasing industrial and agricultural activities that generate anthropogenic inputs (Seeliger and Knak 1982). Although many studies have been carried out to investigate the accumulation of organic trace pollutants in aquatic organisms, generally, no standardized methods were used. Since the levels of hydrophobic contaminants in the water phase are usually too low for reliable quantification, it is difficult to study bioconcentration in the field.

When fishes are exposed to high level of metal ions in aquatic environment, their tissues tend to take up these metal ions through various routes from their surroundings. There are two main routes of metal acquisition; directly from the water and from the diet (Bury et al. 2003). Copper enter in the body of fish through gills after binding to the mucus layer. Accumulation and subcellular distribution of metals in *Nassarius reticulatus*, showed that the gill appeared as main target organ for accumulation of copper and in unexposed tissues, most of

the copper present was found in mitochondrial and microsomal pools. However, increased copper content as a result of exposure to the metal was largely sequester in the cytosolic compartment and associated with protein of low molecular weight (Kaland et al. 1991).

Histology and histopathology can be used as biomonitoring tools for health in toxicity studies (Meyers and Hendricks 1985). Histological changes provide a rapid method to detect effects of irritants, especially chronic ones, in various tissues and organs (Bernet et al. 1999). Histopathological studies with light microscopy and electron microscopy are necessary for the description and evaluation of potential lesions in aquatic animals exposed to various toxicants (Meyers and Hendricks 1985). Biomarkers are predominantly worldwide-recognized tools for the assessment of pollution impacts in marine environment (Viarengo et al. 2007). One of the great advantages of using histopathological biomarkers in environmental monitoring is that this category of biomarkers allows examining specific target organs, including gills and the liver that are responsible for vital functions, such as respiration and the accumulation and biotransformation of xenobiotics in the fish (Gernhofer et al. 2001). Furthermore, alterations found in these organs are generally easier to identify than functional ones (Fanta et al. 2003) and serve as warning signals of damage to animal health (Hinton and Laurén 1990). More than one tissue may be studied for an assessment of biological effects of a toxicant on localized portions of certain organs and also for an assessment of subsequent derangements (*i.e.* degradations) in

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tissues or cells in other locations and this allows for diagnoses of the observed changes (Adeyemo 2008).

The Asian sea bass is the most important aquacultured fish species in India. Copper sulphate is commonly used under the form of baths as a chemotherapeutic in aquaculture (Carvalho and Fernandes 2006) to treat various external diseases (Rábago-Castro et al. 2006). For example, it can be used as a disinfectant treatment against protozoan ectoparasites of the genus *Cryptocarium* and *Amyloodinium*, as well as against bacteria (Rigos et al. 2001; Chen et al. 2006). Its use is widespread as a routine treatment in sea bass hatcheries.

Water borne metal, copper (Cu), exerts the initial toxic effects at physiologically active sites on the gill surface, interfering directly with the branchial ion transporting function. Although Cu is essential for metabolic processes, it can be extremely toxic to the fish. Copper is known to affect Na<sup>+</sup> homeostasis (Matsuo et al. 2005). The gills are multifunctional; given their large surface area, they are responsible for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion, which make them extremely sensitive to water contamination (Au 2004).

The transmission microscopic structure of the gill in the fish exposed to various chemicals has been reported in earlier works (Maina 1990; Pfeiffer et al. 1997; Zahra khoshnood et al. 2011; Ba-Omar et al. 2011). The teleost liver has been shown to be very sensitive to pollutant exposure (Pinkney et al. 2004; Blazer et al. 2007). Biotransformation of organic xenobiotics, excretion of harmful trace metals, food digestion and storage, and the metabolism of sex hormones are the main hepatic functions (Hinton et al. 2001). Therefore, tend to accumulate in the fish liver, exposing the tissues of this organ to comparatively higher levels than those experienced by others (Heath 1995). Ultrastructural changes in the liver have been used as biomarkers of toxic chemicals in environmental risk assessment (Alazemi et al. 1996). The ultrastructure of the fish liver has proved to be valuable as a sensitive indicator of toxicant-induced injury (Braunbeck and Volkl 1991). The structure of tissues can be altered even when the waterborne contaminant is at a low level and hence histopathological assays may provide a valuable screening method before severe damage occurs (Jiraungkoorskul et al. 2007). The toxic effects of cadmium have been studied in the marine fish (Yamawaki et al. 1986; Reid and McDonald 1988; Lemaire-Gony and Lemaire 1992; Thophan et al. 2003, 2004), but there is no sufficient information regarding copper.

The present study was conducted to determine the acute and sublethal toxicity of the heavy metal copper by a static bioassay procedure exposing the fish Asian sea bass, *L. Calcarifer*. The Asian sea bass is considered as an important candidate fish species for brackish water aquaculture. Sea bass is a euryhaline fish and extensively cultured both in earthen ponds and open cages under marine, brackish water and also in freshwater conditions (Barlow et al. 1993). In India, various research programmes have been undertaken on the sea bass in order to evaluate the rearing techniques (Kailasam et al. 2006, 2007; Mohanraj et al. 2013) and also the interest to farm sea bass is increasing day by day.

Various stages of this important fish during the course of their life cycle have to experience a variety of environmental

contaminants in the aquatic ecosystem. With this background, we investigate the impact of copper on the structural modifications of the gill and liver tissues in the Asian sea bass.

## 2 Materials and methods

### 2.1 Experimental fish

A healthy hatchery rearing three month old juvenile Asian sea bass, *L. calcarifer* with a mean total length of  $7.06 \pm 0.15$  cm and a mean total weight of  $10.18 \pm 0.24$  g was obtained from the Rajiv Gandhi Centre for Aquaculture, Thirumullaivasal near Sirkali, Nagapattinam Dist, Tamil Nadu, India. Fish samples were acclimated for 2 weeks in a stock tank to the experimental glass aquaria ( $120 \times 50 \times 50$  cm) filled with 250 L of water with a salinity of  $27 \pm 2$  ppt, under a natural photoperiod of 12 h:12 h (light: dark) cycle. The water in the tanks was passed through a 1- $\mu$ m filter, UV-sterilized, and refilled every day. Fishes were fed twice daily with commercially prepared sea bass pettet feed which contains 2.5 mg kg<sup>-1</sup> of copper. They were starved for 24 h before and during experiment.

### 2.2 Chemical

For preparation of stock solution 3.9 g of Copper II sulphate pentahydrate (CuSO<sub>4</sub>·5 H<sub>2</sub>O (Merck) was dissolved in one litre of double distilled water and used as stock solution. It was stored in a clean standard flask at room temperature, in the laboratory.

### 2.3 Experimental procedures

#### 2.3.1 Test concentration

Short-term sublethal tests were used to evaluate the toxicity of copper in Asian sea bass. This method was developed by the US Environmental Protection Agency. Fishes were exposed to 6.83 and 13.66 ppm sublethal concentration of copper. Doses were theoretically sublethal, at 10% and 20% respectively, of the Maximum Acceptable Toxicant Concentration (MATC), which was 68.3 ppm. The MATC was represented as NOEC (No-Observed Effect Concentration) <MATC <LOEC (Lowest Observed-Effect Concentration). The test concentration was estimated using the application factor (AF) concept, by dividing the limits (NOEC and LOEC) of the MATC by the 96-h LC<sub>50</sub> (AF = MATC/LC<sub>50</sub> = (NOEC – LOEC)/LC<sub>50</sub>).

#### 2.3.2 System design

A recirculation closed system was set up according to Muthuwan (1998). The experiment was carried out in 360 L glass aquarium ( $120 \times 60 \times 50$  cm), in which one compartment ( $50 \times 50 \times 40$  cm) was partitioned by a plastic gauze (mesh

**Table 1.** Water quality characteristics and actual copper concentration during sublethal exposure to Asian sea bass, *L. calcarifer*.

Parameters	Range	Mean + S.D
Dissolved oxygen (mg L <sup>-1</sup> )	6.6–7.1	6.87 ± 0.25
Temperature (°C)	25.6–27.9	26.8 ± 1.15
Salinity (%)	27.4–30.2	29.0 ± 1.44
pH	6.81–8.24	7.60 ± 0.71
Ammonia nitrogen (mg L <sup>-1</sup> )	0.02–0.94	0.57 ± 0.48
Nitrite nitrogen (mg L <sup>-1</sup> )	0.03–0.97	0.58 ± 0.49
Nitrate nitrogen (mg L <sup>-1</sup> )	0.69–0.91	0.81 ± 0.11
Actual copper concentration (mg L <sup>-1</sup> )	0.013–0.026	0.019 ± 0.01

size 1.5 mm) to contain a biofilter. Each aquarium was filled with 300 L of natural sea water (salinity of  $27 \pm 2$  ppt), which was pumped continuously over a biofilter column at a rate of 4 l/min. The water was continuously aerated throughout the experiment.

### 2.3.3 Test procedure

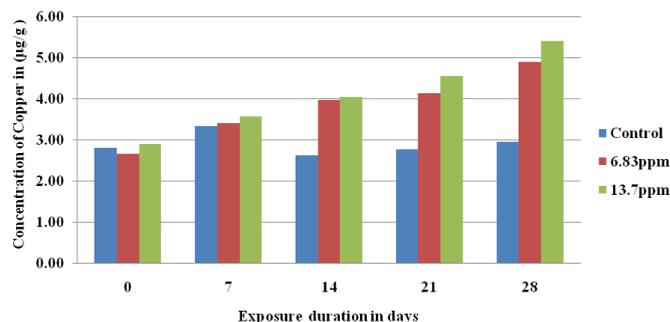
After 2 weeks of acclimatization in a holding tank, ten healthy fish ( $8.06 \pm 0.19$  cm in length and  $11.18 \pm 0.67$  g in weight) were transferred to each aquarium at a loading density of 0.69 g/L. Three replicates were performed for test concentration and control. Fishes were fed twice daily with chopped fresh fish at 10:00 and 14:00 h. Uneaten food was quickly removed from the system. Fishes were starved for 24 h before sampling. The experimental water (50%) was changed every 2 weeks to keep the water quality within acceptable limits according to APHA (1995); water quality (dissolved oxygen, temperature, pH and salinity) was measured everyday and water chemistry (ammonia nitrogen, nitrite nitrogen, nitrate nitrogen) was measured twice weekly. All chemical parameters were determined following the techniques of APHA (1995) using analytical grade reagents. The actual concentration of copper was measured weekly before and after its addition to maintain concentrations at the designed level. Water characteristics and the actual copper concentrations are shown in Table 1. Mortality and behaviour were observed everyday in each concentration. Two fishes from each aquarium were sampled at 0, 7 and 28 days post-exposure.

### 2.3.4 Metal analysis

Prior to metal analysis, gills and liver were dissected and stored in vials. Then the samples were dried in an oven at 80 °C for 24 h and grinded. About 2 gm of powdered samples were taken for digestion. Digestion was performed with 2:1 ratio of concentrated nitric acid and hydrogen peroxide. The heavy metals in the digested samples were determined by atomic absorption spectrophotometer (AAS).

### 2.3.5 Transmission electron microscopy

Fish were quickly anesthetized with 50 mg L<sup>-1</sup> MS 222 (tricaine methane sulfonate) for 2–3 min. The gills and liver

**Fig. 1.** Accumulation of copper in gills of *Lates ealearifer* exposed to sublethal concentrations of copper.

were rapidly removed and processed routinely for transmission electron microscopic study. Gills and liver tissues were cut into small pieces of 1 mm thick and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C, after which the tissues were removed and post fixed in buffered 1% osmium tetroxide for 1 h at 4 °C. Post fixed tissues were rinsed in filtered water (cold) and dehydrated through a graded series of ethanol. Then they were infiltrated with propylene oxide/resin and embedded in resin. Ultrathin sections (250–500 Å) were cut using glass knives, collected on naked copper-meshed grids, and stained with uranyl acetate and lead citrate. The sections were examined and viewed using a Hitachi H-300 electron microscope operated at 50 kV.

## 3 Results

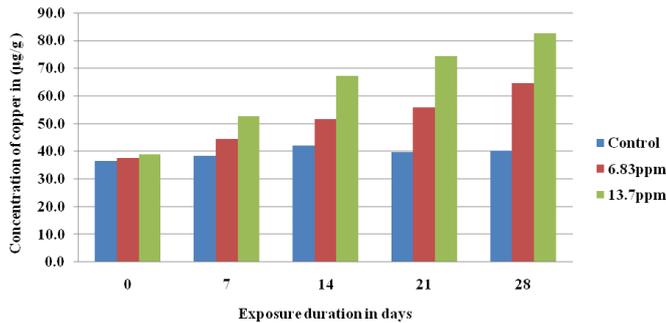
### 3.1 Visual observations

#### 3.1.1 Sublethal exposure

Control fish swam normally, without signs of any abnormality. Some of the fish exposed to low-concentration copper exhibited slight reductions in feeding activity during the second and third weeks. The fish whose copper exposure was high did not swim actively and had reduced feeding activity throughout the exposure period. These signs were related to the time of exposure. Mortality did not occur in the control or in the copper -exposed groups.

### 3.2 Bioaccumulation

Copper accumulation in gills and liver of *L. calcarifer* showed significant variations (Figs. 1 and 2). In the gills of untreated *L. calcarifer*, the copper concentration was between  $2.64 \pm 0.02$  µg/g and  $3.31 \pm 0.02$  µg/g. A sharp increase in copper content was recorded after 28 days of exposure. It was  $4.89 \pm 0.02$  µg/g at 6.83 ppm and  $5.38 \pm 0.02$  µg/g at 13.66 ppm concentrations of copper. In the liver of untreated *L. calcarifer*, the copper concentration was between  $36.50 \pm 0.15$  µg/g and  $42.1 \pm 0.31$  µg/g. At the end of the experiment (28 days), the copper concentration of liver increased to  $64.7 \pm 0.15$  µg/g at 6.83 ppm and  $82.6 \pm 0.15$  µg/g at 13.66 ppm. The results demonstrate that the concentrations of copper were



**Fig. 2.** Accumulation of copper in liver of *Lates calcarifer* exposed to sublethal concentrations of copper.

higher in the liver followed by gills. Metal accumulation varied significantly in tissues of sea bass between different concentrations and periods ( $p < 0.05$ ).

### 3.3 Ultrastructural observations

#### 3.3.1 Gills

##### Control

Under Transmission Electron Microscopy, the gill epithelium of control sea bass was formed by a filament multilayered epithelium, periodically sectioned by longitudinal capillary axes that originated in the lamellae, which were covered by a 2-layered epithelium. Each lamella possessed a central vascular axis, the endothelium of which was composed of pillar cell (PC) cytoplasmic extensions, externally coated with a basal lamina and a loose interstitial space. The superficial layer of the filament epithelium contained mucous cells (MC), chloride cells (CC), their precursors, and intercalating support cells (SC), which were externally covered by a monolayer of pavement cells (PC). One to two erythrocytes are usually recognized within each capillary lumen. Chloride cells are identified as large epithelial cells with light cytoplasm, usually present at the base of lamellae. Mucous cells and pavement cells are also present in the epithelium of the filament and at the base of lamellae, but they lack the light cytoplasm and are smaller than chloride cells (Plate 1, Fig. A).

##### Treated Sub lethal Exposure

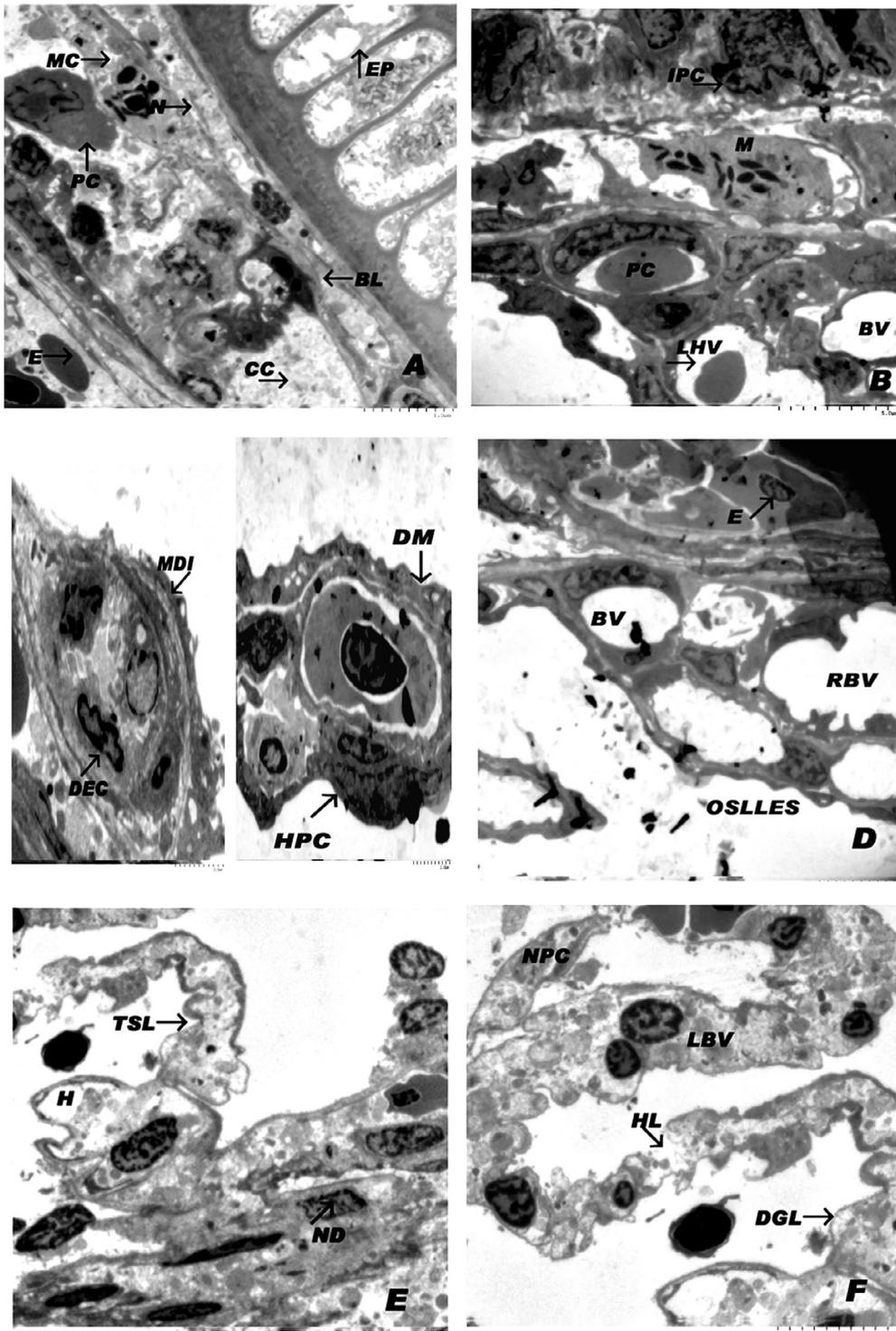
Ultrastructural alterations were noticed in the gills of copper treated fishes. Secondary gill lamellae exhibited hypertrophy and hyperplasia of the epithelial cells. The pavement cell appeared irregular with a considerable loss of microridges. Vasodilatation in many areas of the secondary lamellae with breakdown of the pillar cell system appeared by degenerative and necrotic changes of the pillar cells (Plate 1, Fig. B). Occasionally, proliferation of chloride cells and mucous cells could be identified in the secondary lamella. The chloride cells appeared with dilated vesicles within the cytoplasm and damaged mitochondria, while the mucous cells were completely filled with electron-dense mucous containing vacuoles and no other organelles could be visible in this cell. It is worth mentioning that electron microscopic alterations observed in the

gills during this study were the initial part of the protruding lamellae showing oedema of the interstitial tissue and irregular capillary shapes, whereas vasodilatation was mostly confined to the lamellar basal region and was associated with stretched pavement cells and large hydropic vacuoles were observed at a 6.83 ppm concentration of copper after 7 days of exposure (Plate 1, Fig. B). At lowest concentration of 28 days of copper treatment the enlarged filament intercellular spaces contained macrophage like cells, leucocyte-like cells, and macrophages with large digestive vacuoles (MDI) which frequently showed autolysis (Plate 1, Fig. C). Numerous macrophages or apoptotic bodies were evident on exposure to 7 days of highest concentration of copper. The external cover of pavement cells also exhibited some modifications. These cells rounded up and partially detached, resulting in coalescence and rupture of blood vessels (RBV) (Plate 1, Fig. D). Under a higher magnification of TEM, hypertrophic pavement cells with irregular shape, long cytoplasmic processes and without microridges were observed. The gills showed extensive hypertrophy and hyperplasia of epithelial and chloride cells due to complete fusion of secondary lamellae (Plate 1, Fig. D). Thinning of the epithelial cells of secondary lamellae was highly evident on exposure to 7 days of higher concentration of copper. Moreover, congestion of blood vessels by erythrocytes in the presence of different leucocytes has been observed. Dilatation of the blood vessel walls allows haemorrhage at higher concentrations of copper exposure (Plate 8, Fig. E). Ultrastructurally, the Chloride cells (CC) possessed dilated vesicles within the cytoplasm and damaged mitochondria with cristae regression; the apical pits of CCs appeared enlarged after 7 days of exposure to higher dosages of copper. After 28 days of exposure at higher concentrations of copper, the filament epithelium was extremely reduced in thickness showing an almost complete absence of chloride cells (CC). In the basal region of lamellae, the vascular axis showed extensive vasodilatation with stretching and necrosis of pillar cells (NPC). Due to marked interstitial oedema, large epithelial cell spaces were formed. This progressively leads to lifting of the epithelium up to the tip of the lamellae. The enlarged filament intercellular spaces contained undifferentiated cells, leucocytes, haemorrhagic residues and macrophages with large digestive vacuoles which frequently showed autolysis. At higher concentrations of copper exposure swelling of the lamellar epithelium was seen frequently in surfaces leading to wrinkled and non-homogenous surfaces. Detachment of the lamellar epithelial layer (DEL) was clearly evident after 28 days of exposure to copper in the treated sea bass (Plate 1, Fig. F). The gills showed extensive aneurism with some ruptures in many secondary lamellae and the breakdown of the pillar cell system was seen.

#### 3.3.2 Liver

##### Control

In the untreated fish all the hepatic cells were normal. Ultrastructurally, hepatocytes showed a single rounded nucleus, usually centrally located. The chromatin was granular, with more condensed heterochromatin located at the periphery of the nucleus. The nucleolus was more homogenous and presents high electronic density. The rough endoplasmic reticulum (RER) is arranged in parallel stacks embracing the



**Plate 1.** Transmission Electron micrograph of gill in *L. calcarifer*. (A) Control. (B) After 7 days of exposure to 6.83 ppm concentration of copper. (C) After 7 days of exposure to 6.83 ppm concentration of copper. (D) After 7 days of exposure to 13.66 ppm concentration of copper. (E) and (F) - After 28 days of exposure to 13.66 ppm concentration of copper. Abbreviations used: (PC) Pavement cell. (MC) Mucus cell. (BL) Basal lamina. (E) Erythrocyte. (CC) Chloride cell. (EP) Epithelium. (M) Mitochondria. (N) Nucleus. (BV) Blood Vessel. (PC) Pavement cell. (H) Haemorrhage. (HL) Hypertrophy of lamellae. (ND) Nuclear degenerations. (NPC) Necrotic Pillar cell. (IPC) Irregular shape of Pavement cell. (DM) Damaged mitochondria. (HPC) Hypertrophic pavement cell. (LHV) Large hydropic vacuole. (RBV) Rupture of blood vessel. (MDI) A macrophage with dark inclusions in the secondary lamellae. (DEC) Damaged Epithelial cell. (OSLES) Oedematous secondary lamellae with large epithelial space. (TSL) Thinning of secondary lamellae. (DEL) Detachment of epithelial layer. (LBV) Leucocytes in blood vessel.

nuclear membrane. Both circular and elongated mitochondria were associated to the rough endoplasmic reticulum. Numerous cytoplasmic vacuoles of various sizes and shapes are distributed throughout the cytoplasm, often in close association with the RER. Smooth endoplasmic reticulum (SER) was almost absent. Large amounts of scattered glycogen like particles fill most of the cytoplasm (Plate 2, Fig. A).

#### Treated Sub lethal Exposure

At lowest concentrations of copper after 7 days of exposure in sea bass the main alterations in the structural architecture were concerned with rough endoplasmic reticulum (RER) and mitochondria (M). With comparison to hepatocytes of the control fish, the smooth endoplasmic reticulum (SER) was highly developed; the changes observed were; degranulation and fragmentation of RER (FRER), dilatation and vesiculation of the reticulum cisternae; some hepatocyte nuclei exhibited chromatin clumping (CC) (Plate 2, Fig. B). Flattened stack-like cisternae modified to numerous vesicles due to fragmentation. The most frequent pathological modifications were clearly depicted by the organelle mitochondria. The mitochondrial reactions to copper were swelling, disappearance of cristae, vacuolization, formation of myelinoid-bodies, and the hepatocytes showed massive swollen mitochondria with a loss of cristae and condensed mitochondria. In some cells the nucleus has been pushed to the periphery of the cell instead of the central position exhibiting evidently at 28 days of lower concentrations of copper (Plate 2, Fig. C). After exposure of 7 days at higher concentrations of copper, hydropic swelling of hepatocytes with nuclear pyknosis and chromatin condensation was observed. With reference to storage vesicles, there appeared to be an increase in the lipid droplets (lipidosis, steatosis), within many hepatocytes. The nuclei also showed alterations with dilation of the nuclear envelope and an accumulation of heterochromatin. A slight accumulation of dark minute granules (DMG) was observed in some hepatocytes (Plate 2, Figs. D and E). After 28 days of exposure in higher dosage the hepatocytes showed diffuse degenerative vacuolation (cellular oedema or acute cell swelling) and cytoplasm rarefaction. In some instances myelinoid bodies (MB) were also observed. The nuclei were affected by exposure, showing dilatation of nuclear envelope, rarefaction of karyoplasm and lipid inclusions and complete damage of mitochondria. TEM observations showed severe lesions in hepatocytes (Plate 2, Fig. F).

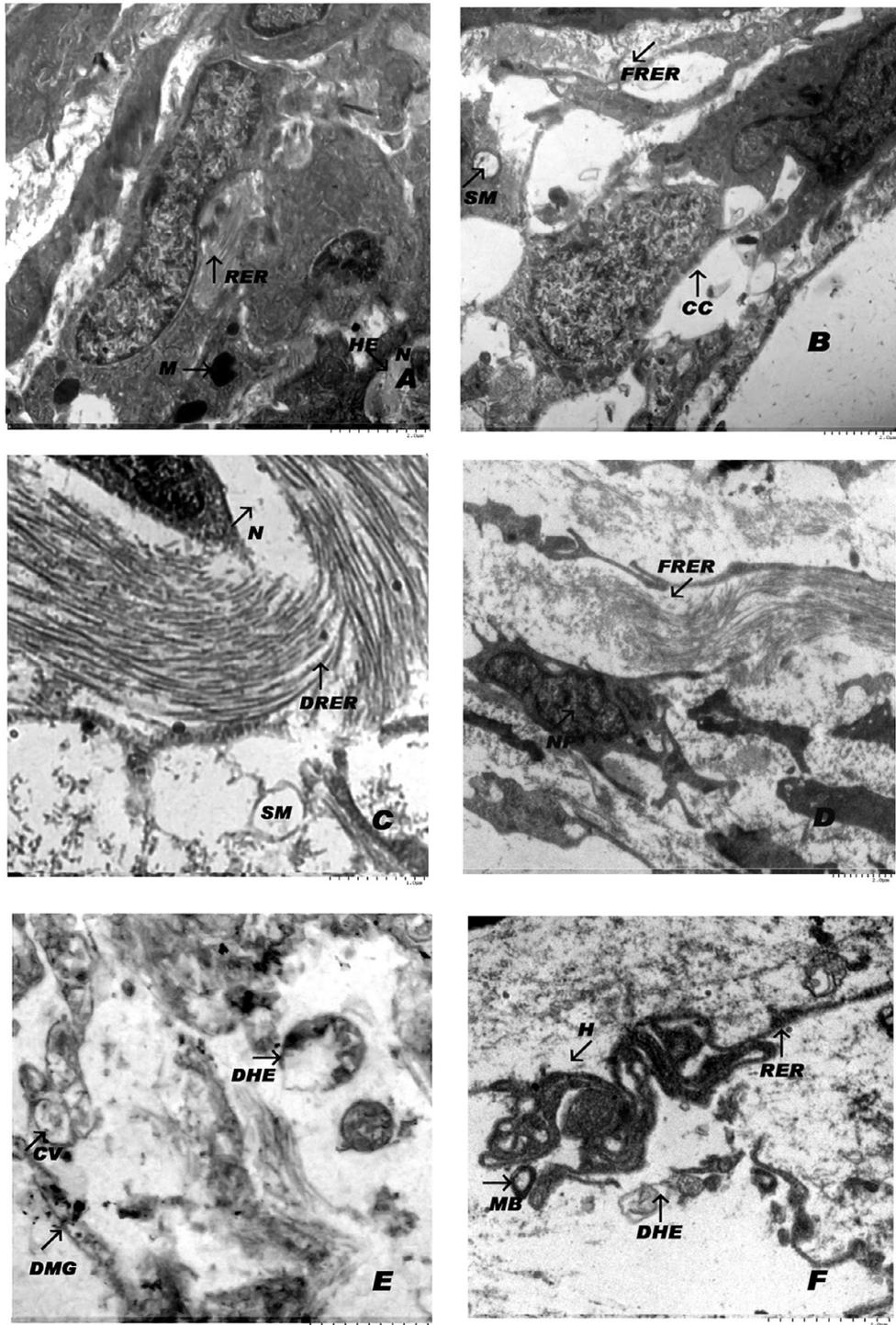
## 4 Discussion

The accumulation of heavy metals by aquatic organisms involves tissues that serve as the site for uptake and absorption like gills, skin and intestine. Among these tissues gills have the ability to concentrate metals and therefore exhibit relatively high potentials for accumulation. Present investigation revealed that fingerlings of *L. calcarifer* were exposed to two sublethal concentrations of waterborne copper for four weeks only. During this exposure time fish has already accumulated significant level of copper in their various tissues. Significant copper accumulation has higher in liver than in gill, in agreement with Abdel-Tawwab et al. (2007). In general, different tissues show different capacities for accumulating heavy metals. Tekin-Ozan and Kir (2008) pointed out that the highest

metal concentrations were found in liver and gills, and muscle tended to accumulate less metal. Arellano et al. (1999) suggested that the copper concentration in liver was higher than that in gills in *Senegales sole*. Present study also indicated that increasing water borne copper concentration significantly increased copper accumulation in all tissues with the exception of vertebrae.

Tissue changes in test organisms exposed to experimental concentrations of toxicants are functional responses that provide information on the mode of action of the toxicant on them. The gills, which participate in many important functions in the fish, such as respiration, osmoregulation and excretion, remain in close contact with the external environment, and particularly sensitive to changes in the quality of the water, are considered the primary target of the contaminants (Poleksic and Mitrovic-Tutundzic 1994; Mazon et al. 2002; Fernandes and Mazon 2003). The ultrastructure of untreated *L. calcarifer*, gill is shown in Plate 1 (Fig. A). The centrally located nucleus normally exhibits little heterochromatin. The cell organelles mitochondria smooth endoplasmic reticulum (SER), rough endoplasmic reticulum ((RER), Peroxisomes and Golgi complex were scattered in the cytoplasm. Gills of treated sea bass exposed to higher sublethal concentrations of copper showed abundant distribution of cytoplasmic vacuoles as well as alterations of cytoplasmic organelles including mitochondria, SER, RER and Golgi complex. Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae are examples of defence mechanisms, since, in general, these result in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants (Mallatt 1985; Hinton and Lauren 1990; Poleksic and Mitrovic-Tutundzic 1994; Fernandes and Mazon 2003). Most part of the gill lesions caused by sublethal exposures affects the lamellar epithelium (Hinton and Lauren 1990); however, some alterations in blood vessels may also occur, when fishes suffer a more severe type of stress. In this study the damaged pillar cells can result in an increased blood flow inside the lamellae, causing dilation of the marginal channel, blood congestion or even an aneurysm (Takashima and Hibiya 1995; Rosety Rodriguez et al. 2002). The formation of aneurysm is related to the rupture of the pillar cells (Heath 1987; Martinez et al. 2004) due to a bigger flow of blood or even because of the direct effects of contaminants on these cells.

Morphologic alterations of the pillar cells can have several secondary consequences in *L. calcarifer* (Plate 1, Fig. B). These cells control the blood pressure of the fish, and changes in the blood pressure and flow can affect the number of irrigated lamellae, the distribution of the blood within the lamellae, the permeability of the branchial epithelium and, as a consequence, the osmoregulatory and gaseous exchange mechanisms (Randall 1982), causing several physiological disorders. The deformation of erythrocytes was obvious, and has possibly reduced the capacity of oxygen transport, consequently causing a certain level of hypoxia. Consequently, the experimental fish tries to compensate the lower levels of oxygen in its tissue by an increase of the respiratory frequency, as the same was observed in *M. gulio*. This is observed not only after intoxication with chemicals, but always when



**Plate 2.** Transmission Electron micrograph of liver in *L. calcarifer*. (A) Control. (B) After 7 days of exposure to 6.83 ppm concentration of copper. (C) After 28 days of exposure to 6.83 ppm concentration of copper. (D) and (E) After 7 days of exposure to 13.66 ppm concentration of copper. (F) After 28 days of exposure to 13.66 ppm concentration of copper. Abbreviations used: (RER) Rough Endoplasmic Reticulum. (M) Mitochondria. (HE) Hepatocyte. (N) Nucleus. (FRER) Fragmentation of Rough Endoplasmic reticulum. (SM) Swollen of mitochondria. (CC) Chromatin clumping in nucleus. (DRER) Damaged Rough Endoplasmic reticulum. (NP) Nuclear Pyknosis. (CV) Cytoplasmic vacuolation. (MB) Myelinoid bodies. (DHE) Degenerated hepatocyte in mitochondria. (H) Hepatocytes shows dilation and fragmentation of RER. (DMG) Dark minute granules.

there is a change in the respiratory lamellae, caused by any environmental changes (Fanta-Feofiloff et al. 1986; Fanta et al. 1989, 1995). Several other studies have shown similar effects of pesticides on fish gills in accordance with our present study (Cengiz and Unlu 2002, 2003). Haemorrhage in the primary and secondary gill lamellae, degeneration and necrosis of epithelial cells, distortion of the secondary lamellae, and disruption of epithelial cells from pillar cells were observed in gill tissues of *Anabas testudineus* (Santhakumar et al. 2001) similar to the present study. Degenerative changes in gills, such as detachment and lifting of the epithelial linings from the surface of the gills, uncontrolled regeneration of the primary lamellae and secondary lamellae, hypertrophy, hyperplasia, necrosis of the epithelial cells, dilation of the blood sinuses of the secondary lamellae, lamellar aneurysm, haemorrhages etc., were noticed after exposure to sublethal concentrations of profenofos (Rao 2006). Coutinho and Gokhale (2000) found epithelial lifting in the gills of carp (*C. carpio*) and tilapia (*O. mossambicus*) exposed to the effluents of a wastewater treatment plant. According to Mallat (1985) such alterations are non-specific and may be induced by different types of contaminant. As a consequence of the increased distance between water and blood due to epithelial lifting, the oxygen uptake is impaired. Toxic substances can injure gills, thus reducing the oxygen consumption and disrupting the osmoregulatory function of aquatic organisms (Saravana Bhavan and Geraldine et al. 2000). However, fishes have the capacity to increase their ventilation rate, to compensate low oxygen uptake (Fernandes and Mazon 2003). Histopathological changes in the gill tissue of the fish *C. punctata* exposed to sublethal concentrations of Butachlor and Machete, an Herbicide were bulging of tips of primary gill filaments and fusion of disorganized secondary gill filaments (Tilak et al. 2005) Similarly the toxic sublethal concentrations of the fenvalerate technical grade in the gill of *C. mrigala* was evaluated and was found to have marked pathological changes such as necrosis, progressive degeneration in the gill tissue (Anita Susan and Tilak 2003).

The present electron micrographs show a reduction in the quantity of the microridges of the pavement cells in the gills of *L. calcarifer*. Such reduction was also observed by Wong Chris and Wong (2000) and Mazon et al. (2002). Mallat (1985) suggested that the microridges are related to the retention of mucous on the epithelium as a way to protect it against environmental alterations. These pathological changes may be a reaction to toxicant intake or an adaptive response to present the entry of the pollutants through the gill surface (Mohamed 2009). The damages observed in the gills in terms of hypertrophy, fusion of secondary lamellae and necrosis could cause a decrease in free gas exchange, thus affecting the general health of the fish (Skidmore and Tovell 1972). The ultra structural alterations were more severe and progressive in high concentration copper-treated gills (Plate 1, Figs. E and F). The gills, because of their direct and permanent contact with water, are primary and very sensitive receptor surface for aquatic pollution (Wood and Soivio 1991; Bernet et al. 1999) and have been widely used to investigate the toxicity effects from heavy metals (Wood 1992). The severe morphological anomalies of gill observed in the present survey are non-specific and occurred under different exposure

conditions (Evans 1987; Alazemi et al. 1996; Neskovic et al. 1996; Haaparanta et al. 1997; Teh et al. 1997; Oliveira Ribeiro et al. 2000; Roberts and Rodger 2001; Maharajan et al. 2012a, 2012b).

The teleost liver is one of the most sensitive organs to show alteration in biochemistry, physiology and structure following exposure to various types of environmental pollutants (Hinton and Couch 1998). Since the liver is the central site of numerous vital functions (i.e. basic metabolism, bile production, vitellogenesis) and accumulation, biotransformation and excretion of organic and inorganic contaminants, the hepatocytes are powerful sources of biomarkers (Braunbeck 1998; Bernet et al. 1999). The deterioration of the regular compartmentation of the cytoplasm is a very early and unspecific signal of disturbance of hepatocellular homeostasis (Braunbeck 1998). The same ultrastructural modifications of the organelles were also observed in the present survey. Some of these changes, such as the proliferation of endoplasmic reticulum (ER), (Plate 2, Figs. B and C) indicate stimulation of defence and regenerative processes linked to detoxification and could be classified as adaptative while others, such as dilatation of the ER and the nuclear envelope, express the onset of degenerative processes in the liver (Braunbeck 1998). The major cytotoxicity mechanism of copper is the alteration of ion and non-electrolyte transport and cell volume regulation, which finally leads to cell swelling (Ballatori and Boyer 1996). With regard to the storage products, the increase in lipid droplets observed in this study and by other authors after lindane, cadmium and terbutylazine exposures (Biagianni-Risbourg et al. 1996; Dezfuli et al. 2006) could be due to the decline of protein synthesis and the consequent non utilization of lipids for lipid-protein conjugation (Cheville 1994). The manifestation of cytopathologic changes herein documented might suggest a severe hepatic dysfunction and the impairment of the physio-metabolic process in *L. calcarifer* liver; this finding is in agreement with the principle of the relationship and mutual interference between structure and function expressed by Hinton et al. (1987).

The histopathological alterations in the liver observed in the present study were sinusoid dilation with blood congestion, hydropic swelling of hepatocytes, and dark granule accumulation. Lipid droplet accumulation in many hepatocytes was observed only in subchronic exposure and severity depended on the time of exposure. These pathologies are consistent with those of previous reports (Gupta and Rajbanshi 1982; Wani and Latey 1983; Rani and Ramamurthi 1989). The cellular damage of the liver was more severe and prominent in a lethal exposure than in a sublethal exposure (Brown et al. 1984). Abundant glycogen content was observed in many hepatocytes at the 28 days exposure of high concentrations of copper in *L. calcarifer*. The result of this study was similar to that of Sastry and Subhadra (1982, 1985) who studied on the chronic effects of Cd ( $0.2 \text{ mg L}^{-1}$ ) in cat fish *H. fossilis* and found that after 60 days of exposure, liver glycogen increased which was related to the decreased activity of lactate dehydrogenases, pyruvate dehydrogenases and succinate dehydrogenase in the liver. A wide spectrum of RER modifications, appearance of myelinoid bodies, deformation of mitochondria and the nuclear envelope are also herbicide induced alterations in hepatocytes of rainbow trout, *O. mykiss*, and carp,

*C. carpio*, as reported respectively by Braunbeck et al. (1992) and Szarek et al. (2000). According to Franchini et al. (1991), the increase in heterochromatin in the nuclei, in addition to the pathological changes within RER, suggests impairment to the synthetic and secretory activities of the cell. With reference to the extensive development of SER in the sea bass exposed to copper in this study, the same phenomenon has been seen in the hepatocytes of this and other fish species when treated with lead, benzopyrene and certain pesticides (Franchini et al. 1991; Braunbeck 1998). Hypertrophy of the ER is a classical response of teleosts to pollutants and is linked to hepatic detoxication mechanisms (Hawkes 1980). The mitochondrial structural alterations noticed within the hepatocytes and enterocytes of mercury-exposed sea bass were commonly found in different organs following toxic injury (Lemaire et al. 1992), but their significance is difficult to evaluate (Biagiatti-Risbourg and Bastide 1995).

The scanning electron microscopy which dealt with surface modifications sea bass that occur after sublethal exposure has proved to be a sensitive indicator of copper induced injury (Paruruckumani et al. 2015). The toxic effects of cadmium have been studied in marine fish (Lemaire-Gony and Lemaire 1992), but relatively few investigations have defined the ultrastructural alterations of important organs (Thophon et al. 2003). Ultrastructural changes in the liver have been used as biomarkers of toxic chemicals in environmental risk assessment (Alazemi et al. 1996). In the present study, the secondary lamella exhibited proliferation of chloride cells and mucous cells. The regular sloughing of mucus from the surface of gills into the media helps to remove the bound pathogens, toxicants and foreign matters which adhere to the gills. Under TEM, the cytoplasmic changes in fish liver like proliferation of endoplasmic reticulum and nuclear envelope were well illustrated than the SEM technique (Paruruckumani et al. 2015).

## 5 Conclusions

In the world, sea bass hatcheries are running successfully even though, in the larval rearing period problems are faced with bacterial and protozoan diseases. To control these diseases, copper sulphate treatment is applied in the hatchery system. Copper accumulates in the tissues of juvenile sea bass and lethal concentration of copper kills them. But they survive in the sublethal concentration as a result of cumulative accumulation. During this study, we have noticed the accumulation of copper in the body tissues and structural changes in the cellular level. In man, consumption of the sea bass will cause serious issues that are specific to the toxicant. This process will damage the organism silently, without causing any immediate changes. The assessment of copper toxicity to sea bass tissues with the aid of ultra microscopy would be highly significant to hatcheries in reducing the usage of copper sulphate and search for alternative control measures. Our study reinforces the importance of histopathology analysis in toxicity control programmes as a tool for a more distinct evaluation of environmental quality. We suggest the hatcheries to avoid or minimize the usage of copper sulphate.

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