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Surface Ultrastructural Changes in the Gill and Liver Tissue of Asian Sea Bass *Lates calcarifer* (Bloch) Exposed to Copper

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Abstract Surface ultrastructure of the gill and liver of 3-month-old Asian sea bass, *Lates calcarifer*, after copper exposure, was investigated by scanning electron microscopy (SEM). Fish samples were exposed to copper concentrations of 6.83 and 13.66 ppm (sublethal) for 28 days with parallel untreated control. These structures showed structural modifications in both low and high concentrations of copper exposure. Oedema, hyperplasia, desquamation, necrosis, epithelial lifting, lamellar fusion, collapsed secondary lamellae, curling of secondary lamellae and aneurism in the secondary lamellae were observed in gill tissues exposed to copper. Hepatic lesions related to cloudy swelling of hepatocytes, congestion, vacuolar degeneration, dilation of sinusoids and nuclear hypertrophy were evident in the exposed sea bass liver tissue.

Keywords Surface ultrastructure · Gills · Liver · Copper · *Lates calcarifer*

Introduction

Copper is one of the most toxic trace metals to marine biota [1] which poses considerable risk to marine ecosystems where human influences have enhanced the natural (background) copper concentrations. Potential risks occur when copper is introduced into marine ecosystems by mining activities, antifouling paints on boats, marinas, ports and jetty pylons, runoff from fungicidal uses (e.g. copper sulphate) and sewage effluent. In aquaculture industry, copper sulphate is used as an algaecide and as a therapeutic chemical for various ectoparasitic and bacterial infections [2, 3]. Excess copper can result in adverse toxicological effects and maybe poisonous to aquatic animals. Fishes are the simple and reliable biomarkers of copper pollution of aquatic bodies [4, 5].

Histopathological investigations have long been recognized as reliable biomarkers of stress in fish [6] and have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, both in laboratory and field studies. The gills [7], liver [8] and kidney [9] are the common primary target organs for many chemicals principally because of their role within the body. The gills are multifunctional—given their large surface area, they are responsible for respiration, osmoregulation, acid–base balance and nitrogenous waste excretion. Thus, they are extremely sensitive to water contamination [10]. Due to their delicate structure, they are liable to damage by any irritant material whether dissolved or suspended in water [11] and respond to environmental changes by structural alterations. In addition, they are not irritant specific but affected by the effect of factors' intensity and duration of exposure, especially in cases of sublethal concentration of pollutants [12–14]. Gills are efficient tools for biomonitoring potential impacts [15, 16] because of their contact with water and high permeability [17–19]. Gills also help know the environmental impact caused by pollutants [20–22].

Fish liver is an interesting model for the study of interactions between environmental factors and hepatic structures and functions. The liver of teleosts is important in the maintenance of internal homeostasis and the metabolism of xenobiotics [23]. It has also been shown to accumulate foreign compounds [24] and be susceptible to damage by toxic agent [25, 26]; the functional integrity of the liver in fish can be

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affected by xenobiotics [27]. According to Buck [28], the liver is the first line of defence against copper poisoning. Copper becomes toxic only when the high binding capacity of the liver exceeds and copper is released into the blood stream. In fish also, the liver is the major storage organ for copper [29, 30], and hence, research on fish liver is important especially that related to aquaculture condition and aquatic pollution-induced problems.

Asian sea bass *Lates calcarifer* are a commercially significant fish in tropical regions. There is a very high economic value and demand for sea bass, but their farming is threatened due to heavy metal toxicity. Only a very few studies have reported in detail on the ultrastructural effects of toxic substances in the respiratory and liver tissues [31–35]. So, through our research, we made an attempt to study surface modifications of the gill and liver tissues in response to copper in Asian sea bass.

Materials and Methods

Experimental Fish

Healthy hatchery reared 3-month-old juvenile Asian sea bass *L. calcarifer* with a mean total length of 7.06±0.15 cm and a mean total weight of 10.18±0.24 g were obtained from the Rajiv Gandhi Centre for Aquaculture, Thirumullaivasal near Sirkali, Nagapattinam Dist, Tamil Nadu, India. Fish samples were acclimatized for 2 weeks in a stock tank to the experimental glass aquaria (120×50×50 cm) filled with 250 l of water with a salinity of 27±2 ppt, under a natural photoperiod 12:12 h (light:dark) cycle. The water in the tanks was passed through a 1-μm filter, UV-sterilized and refilled daily. Fish were fed twice daily with commercially prepared sea bass pellet feed which contains 2.5 mg/kg of copper. They were starved for 24 h before and during experiment.

Chemicals Used

For preparation of stock solution, 3.9 g of copper ll sulphate pentahydrate ($CuSo_4$ 5 H_2O) (Merck) was dissolved in 1 l of double-distilled water and used as stock solution. It was stored in a clean standard flask at room temperature in the laboratory.

Experimental Procedures

Test Concentration

Fish were exposed to nominal 6.83 and 13.66 ppm as copper. Doses were theoretically sublethal, 10 and 20 %, respectively, of the maximum acceptable toxicant concentration (MATC), which was 68.3 ppm. The MATC was represented as no observed effect concentration (NOEC)<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_{50} ($AF=MATC/LC_{50}=(NOEC-LOEC)/LC_{50}$).

System Design

A recirculation closed system was set up according to Muthuwan [36]. The experiment was carried out in 360 l glass aquarium (120×60×50 cm), in which one compartment (50×50×40 cm) was partitioned by a plastic gauze (mesh size 1.5 mm) to contain a biofilter. Each aquarium was filled with 300 l of natural sea water (salinity of 27±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.

Test Procedure

After 2 weeks of acclimatization in a holding tank, ten healthy fish (8.06±0.19 cm in length and 11.18±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. Fish were fed twice daily with chopped fresh fish at 1000 and 1400 hours. Uneaten food was quickly removed from the system. Fish 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 [37]. Water quality (dissolved oxygen, temperature, pH and salinity) was measured daily, and water chemistry (ammonia nitrogen, nitrite nitrogen, nitrate nitrogen) was measured twice weekly using the Merck water quality analyser kit. The ammonia nitrogen and nitrite nitrogen levels were controlled and kept within 0.2 mg/l for exchanging the water in 25 %. The actual concentration of copper was measured weekly before and after its addition to maintain copper concentrations at the designed level. Mortality and behaviour were observed daily in each concentration. Two fish from each aquarium were sampled at 0, 7 and 28 days post-exposure.

Scanning Electron Microscopic Study

Fish were quickly anesthetized with 50 mg/L MS 222 (tricaine methane sulphonate) for 2–3 min. Gills and liver tissues were rapidly removed and processed routinely for scanning electron microscopic studies. Gills and liver tissues were cut into small pieces of 1 mm thickness and fixed in 2.5 % glutaraldehyde prepared in cacodylate (sodium phosphate) buffer adjusted to pH 7.4 for 4 h and afterward washed in phosphate buffer for 15 min. Then, samples were post fixed in 1 % osmium tetroxide for 80 min and washed in phosphate buffer. After dehydration in ascending series of acetone, samples were coated

with gold palladium and observed through scanning electron microscope (LEO Stereoscan, 440).

Results

Visual Observations

Sublethal Exposure

Control fish were good, without any morphological deviation. Some of the fish exposed to low-concentration copper exhibited slight reductions in feeding activity during the second and third weeks. In higher concentrations of copper, the fish did not swim actively and had reduced feeding activity throughout the exposure period. These signs were dosage dependent. Mortality did not occur in control or in copper-exposed groups.

Surface Ultrastructural Observations

Gills

Control In scanning electron microscopic studies, the architectural pattern of gills of *L. calcarifer* is essentially similar to that of the other teleost fish. SEM images provide good three-dimensional views of different regions of respiratory lamellae. There are four gill arches on each side of the buccal cavity. Each arch is composed of numerous gill filaments which have two rows of secondary lamellae that run perpendicular to each filament. The secondary lamellae were sequentially lined up along the two sides of the primary lamella. In control fish, the secondary lamellae constituted evenly spaced parallel plates. The epithelial cells covered both the primary and secondary lamellae. Secondary lamellae are plate-like projections at right angles to the gill filaments. They lie parallel to the adjacent lamellae and covered by a thick and coarse epithelium. Chloride and mucous cells were distributed primarily at the bases of the secondary lamellae. Numerous water pores and mucous cell openings with well-developed microridges are discernible at the lamellae (Plate 1a). Chloride cells, seen as pit openings, are found among the protuberances and cavities of the surface. In high resolution of SEM, the surface epithelium of lamellae shows clear demarcation between cells and microridges.

Treated Sublethal Exposure The gills in the treated fish exhibited noteworthy damages than those in the control. Following the treatment at a 6.83 ppm concentration of copper after 7 days of exposure, diffuse oedema (E) and detachment of the lamellar epithelium (epithelial lifting) with the formation of large subepithelial spaces within the secondary lamellae were observed. SEM examination showed swelling and curling of secondary lamellae (SSL) (Plate 1b). After 28 days of low concentrations of copper, the gills showed extensive aneurism with some ruptures in many secondary lamellae and the breakdown of pillar cell system was seen. Moreover, partial or complete secondary lamellar fusion (FSL) (Plate 1c) and thickening of primary lamellae were encountered in the exposed sea bass as a result of inter-lamellar epithelium and chloride cell hyperplasia. SEM examination also showed complete fusion of secondary lamellae and surface wrinkling in numerous areas as a result of epithelial hyperplasia and/or hypertrophy. The surface wrinkling was severe in some regions. Mucous cell ruptures and a thick blanket of mucus (TBM) secretion (Plate 1d) were seen above the damaged epithelium. At the lowest concentration, also necrosis and leukocyte infiltration (granulocytes and macrophages) in secondary lamellae were observed. Curling of secondary lamellae (CSL) was severe in higher concentrations of copper-treated sea bass (Plate 1d). Lifting of the epithelium was common and particularly severe and extensive after 7 days of high concentrations of copper exposure (Plate 1e). In addition to this, sloughing of the primary and secondary gill epithelium (SGLE) was clearly evident in treated sea bass. The epithelial surface with microridges exhibit damaged and irregular appearance (IAES). Fusion of the secondary gill lamellar epithelium was clearly displayed in high concentrations of copper after 28 days of exposure, and this finally ended with a complete degeneration of secondary lamellae (Plate 1f). Exfoliated epithelium was a common feature in all treated fish, and the secondary lamellae were highly deformed as a consequence of the lifting of the epithelium and severe hyperplasia which led to the conjunction of adjacent filaments. In higher magnification, the SEM picture clearly depicts the denuding of the boundaries of surface epithelial cells of both primary and secondary lamellae. In some regions, the lamellae of treated fish were thinner than those of the control (Plate 1f).

Liver

Control In all control fish, the ultrastructural morphology of hepatocytes was normal. The surface of the liver was covered with serous membrane and some connective tissue extending inward into parenchyma. It was composed of parenchymal cells (hepatocytes) (HC) and lattice fibres, which support the former. Hepatic cells were roundish polygonal, containing clear spherical nucleus (SN). They were located among sinusoids forming cord-like structures known as hepatic cell cords. Hepatic cells have many vital functions. Other than the secretion of bile, they play an important role in protein, lipid and carbohydrate metabolism. They serve as storage sites for some nutrients, and detoxification is another function attributed to them (Plate 2a).

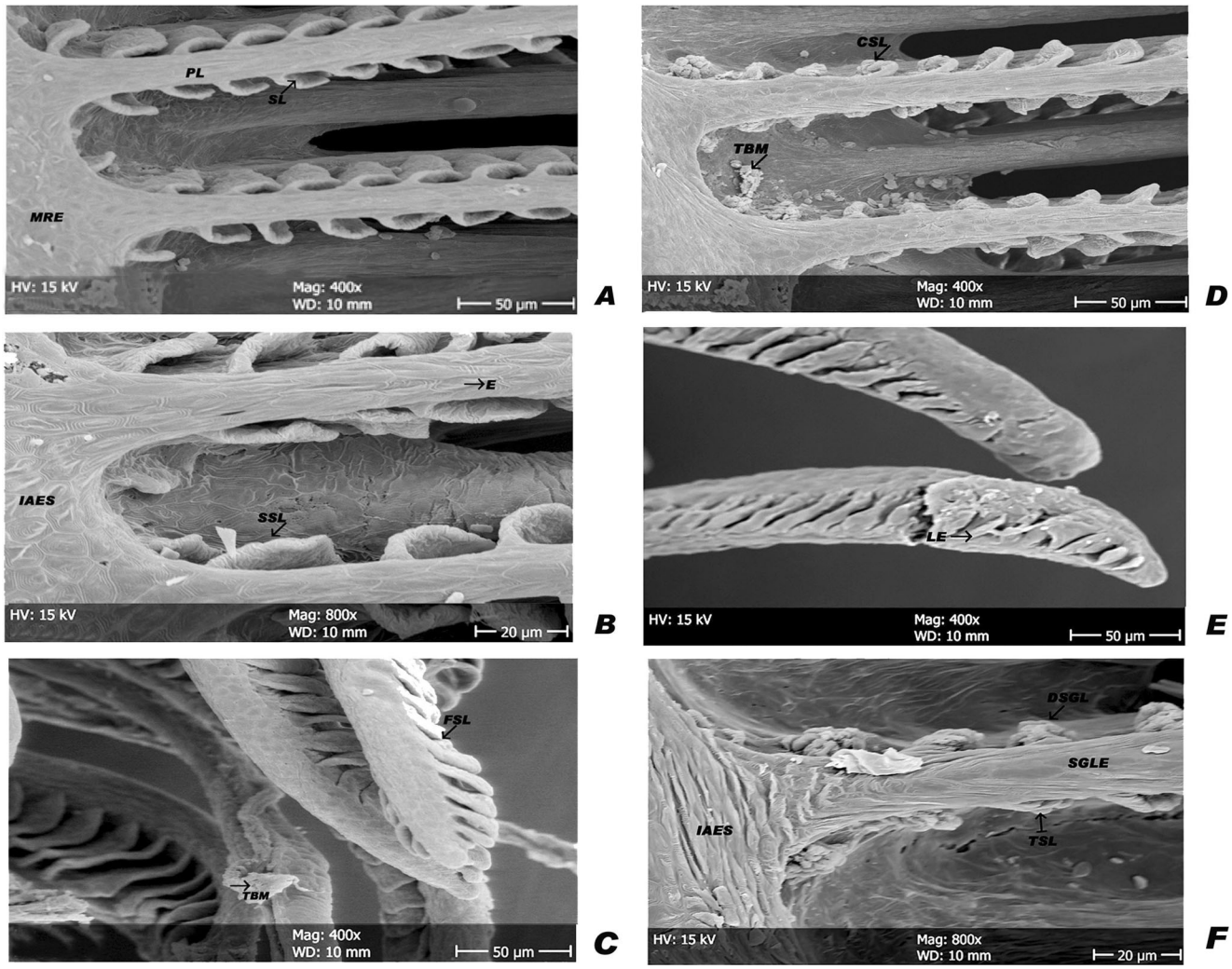

Plate 1 Scanning electron micrograph of gill in *L. calcarifer*. **a** Low magnification of SEM of respiratory lamellae of control fish. **b** Higher magnification SEM of gill tissue exposed to 6.83 ppm concentration of copper and sacrificed after 7 days displayed swelling in the secondary lamellae (SSL) and wrinkled epithelium. **c**, **d** Low magnification of SEM after 28 days of exposure to 6.83 ppm concentration of copper. Note thick blanket of mucus (TBM) and fused secondary lamellae (FSL). **e** SEM of gill tissue after 7 days of exposure to 13.66 ppm concentration of copper showing lifting of epithelium (LE) and note the complete fusion and distortion of secondary lamellae. **f** Higher magnification of SEM of gill tissue after 28 days of exposure to 13.66 ppm concentration of copper showing thinning of secondary lamellae (TSL) and sloughing of epithelial surface. Abbreviations used: *PL* primary lamellae, *SL* secondary lamellae, *MRE* microridged epithelial cell, *IAES* irregular arrangement of epithelial surface, *SSL* swelling of secondary lamellae, *E* oedema, *TBM* thick blanket of mucus, *FSL* fusion of secondary lamellae, *CSL* curling of secondary lamellae, *LE* lifting of epithelium, *DSGL* damaged secondary gill lamellae, *SGLE* sloughing of gill lamellar epithelium, TSL thinning of secondary lamellae

Treated Sublethal Exposure Copper caused severe ultrastructural changes in the liver; the damage severity and its extension increased with the concentration of metal and duration of exposure. In exposed sea bass, the regular compartmentalization was totally lost. Other changes in lower concentrations of copper exposed to 7 and 28 days include cloudy swelling of hepatocytes, congestion, vacuolar degeneration, dilation of sinusoids and nuclear hypertrophy (Plate 2b, c). After 7 and 28 days of higher concentrations of copper exposure, the hepatocytes showed hydropic swelling (Plate 2d). Large lipid droplets and abundant glycogen occupied most of the area of hepatocytes (Plate 2e, f).

Discussion

The scanning electron micrographs of the untreated sea bass gill epithelium revealed normal architecture (Plate 1a). In contrast, the gills of *L. calcarifer* exposed

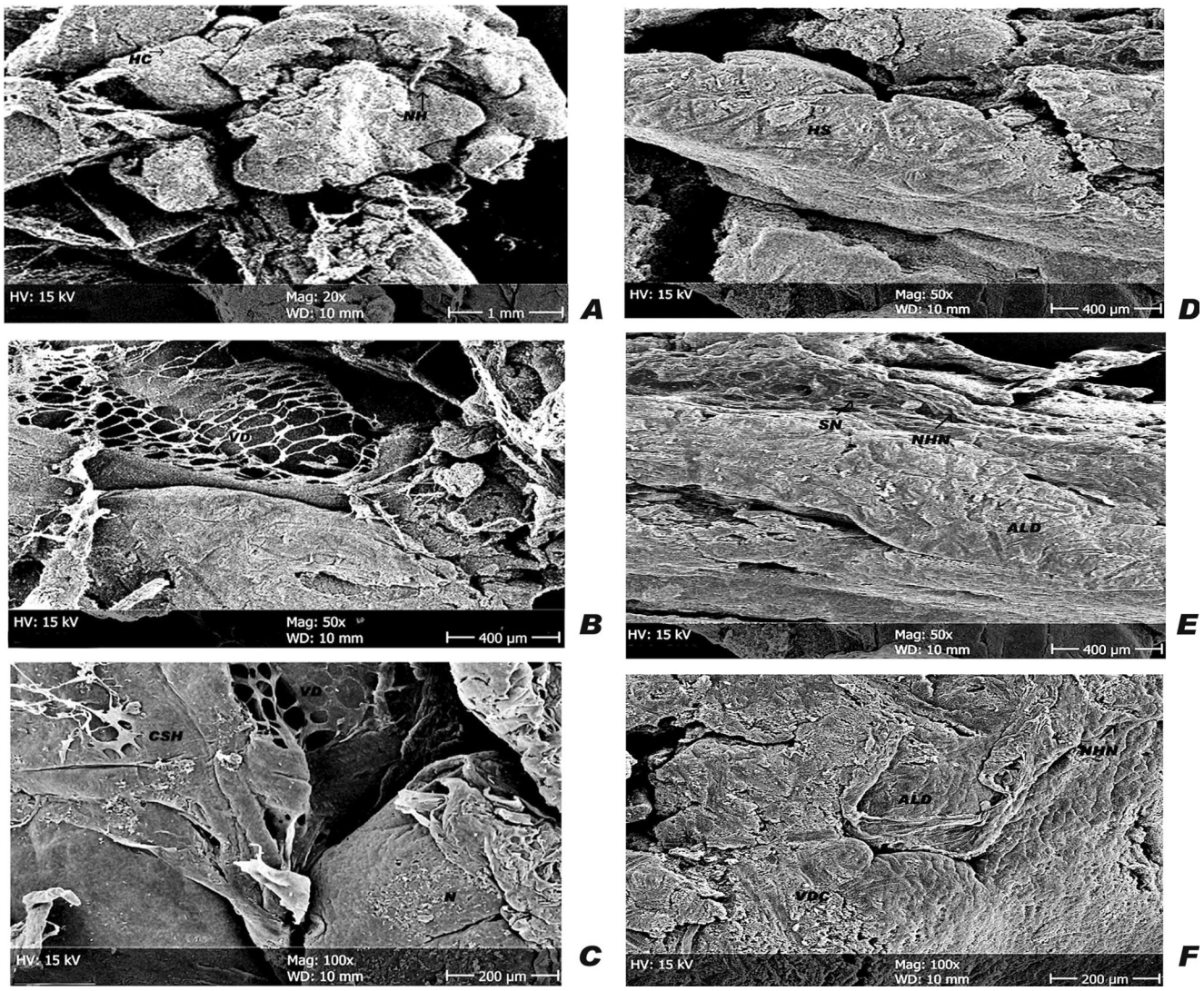

Plate 2 Scanning electron micrograph of liver in *L. calcarifer*. **a** Lower magnification of scanning electron micrographs of the liver of control fish showing normal hepatocytes. **b** SEM of liver tissue after 7 days of exposure to 6.83 ppm concentration of copper showing highly distorted hepatocytes. **c** SEM of liver tissue after 28 days of exposure to 6.83 ppm concentration of copper showing cloudy swelling of hepatocytes (CSH). **d**, **e** SEM photograph of liver tissue after 7 days of exposure to 13.66 ppm concentration of copper depicting necrosis of hepatocyte (NHN) and accumulation of lipid droplets (ALD). **f** Higher magnification of SEM of liver tissue after 28 days of exposure to 13.66 ppm concentration of copper showing vacuolar degeneration (VD) and swelling of hepatocytes. Abbreviations used: *HC* hepatocyte, *HN* hepatocyte nucleus, *VD* vacuolar degeneration, *CSH* cloudy swelling of hepatocytes, *NHN* necrosis of hepatocyte nucleus, *VDC* vacuolar degeneration of cytoplasm, *N* necrosis, *HS* hydrophic swelling, *ALD* accumulation of lipid droplets, *SN* spherical nucleus

to copper during 28 days presented a higher occurrence of histopathological lesions such as hypertrophy, fusion of secondary lamellae, oedema and mucus openings. Numerous types of gill damage have been documented in fish experimentally exposed to toxicants or in populations sampled from polluted environments [7, 38, 39]. Most of the gill histopathological changes are largely non-specific as confirmed by the occurrence of similar alterations under a wide range of toxicant-exposure conditions [40]. Hyperplasia with lamellar fusion, telangiectasia, oedema with epithelial lifting and desquamation, as documented in the present survey, are typical to organochlorines, petroleum compounds, organophosphates, carbamates, herbicides and heavy metals in other animals [41–44] and suggest an impairment to the respiratory and osmoregulatory functioning of the gills [10]. Exposure to heavy metals produces morphological and functional modifications in the branchial epithelium [7, 45]; mercury inhibits gill respiration at sub-acute and acute levels [46].

The first sign of pathology included oedema of epithelial cell in gills. This is due to the epithelium covering the secondary lamellae lifting away in a continuous sheet from the pillar cell system, thus, increasing the diffusion distance from water to blood (Plate 1e). The secondary lamellae showed capillary congestion or aneurism, similar to those reported in *Gnathonemus petersii* exposed to 10 mg/l of cadmium for 6 h [7]. This lamellar aneurism resulted from the collapse of the pillar cell system and the breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward [7]. The hypertrophy and hyperplasia of epithelial and chloride cells with partial or complete fusion of lamellae also occurred in this study (Plate 1c). The pathology related to the chloride cell hyperplasia was to eject the Cd^{2+} absorbed by the gills [47–49].

The immediate morpho-pathological response of the gills of fish exposed to ambient copper is often manifested by a significant increase in the density of its mucous cells (Plate 1c) [50–53]. The large quantity of mucous secretion acts as a defence mechanism with response to toxic substances [54, 55]. The sloughing of mucus from the surface of gills helps to remove the bound pathogens, toxicants and foreign matters [56].

Changes to the ultrastructure of fish livers have proven to be suitable and sensitive signs of toxicant-induced injury and have been used as biomarkers of chemicals in environmental risk assessments [57, 58]. There have been numerous reports on histo-cytopathological modifications in livers of fish exposed to a wide range of organic compounds and heavy metals [10, 41, 59]. The loss of the regular cytoplasmic compartmentation is a typical unspecific ultrastructural reaction of fish hepatocytes which indicates disturbance of hepatocellular homeostasis [57]. Some of the alterations observed in the hepatic cells in the present study, such as vacuolar degeneration, dilation of ER and lipid droplet accumulation, are consistent with those documented in specimens of *Dicentrarcus labrax*, *L. calcarifer* and *Carassius carassius* acutely treated with lead and cadmium [39, 44, 60–62]. A massive enhancement in the number of lipid droplets in the present study resulted from the decline of protein synthesis, and this accompanies with cellular injury. Glycogen deposition resulted in the disintegration of cellular organelles, and this may disturb the metabolic pathways of hepatocytes [63].

Copper reaches water bodies during treatment of the disease of fish and the control of algal blooms in sea bass hatchery. These copper will accumulate in their tissues of juvenile sea bass. Lethal concentration of copper can kill the fish. When fishes suffer from sublethal effects as a result of cumulative accumulation of copper, they may survive for longer times. In man, a daily consumption of these fishes will cause the ill effects that are specific to the toxicant. This process will damage the organism silently, without causing any immediate abrupt changes. The changes may be at genetic levels inducing genotoxicity, but concerted effort in reducing the use of copper and implementing natural remedies for disease control in sea bass hatchery can help resolve the problem of heavy metal pollution.

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