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ORIGINAL ARTICLE

Ultrastructural effects on gill tissues induced in red tilapia *Oreochromis* sp. by a waterborne lead exposure

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Abstract Experiments on hybrid red tilapia *Oreochromis* sp. were conducted to assess histopathological effects induced in gill tissues of 96 h exposure to waterborne lead (5.5 mg/L). These tissues were investigated by light and scanning electron microscopy. Results showed that structural design of gill tissues was noticeably disrupted. Major symptoms were changes of epithelial cells, fusion in adjacent secondary lamellae, hypertrophy and hyperplasia of chloride cells and coagulate necrosis in pavement cells with disappearance of its microridges. Electron microscopic X-ray microanalysis of fish gills exposed to sublethal lead revealed that lead accumulated on the surface of the gill lamella. This study confirmed that lead exposure incited a difference of histological impairment in fish, supporting environmental watch over aquatic systems when polluted by lead.
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1. Introduction

Metal pollution has been an environmental issue in many developed and developing countries for decades, and there is a substantial need to understand the bioaccumulation and toxicity of metals in aquatic organisms (Wang and Rainbow, 2008).

Minerals, including some metals have an important and key role in the evolution of Biochemistry. Some trace elements that are known to perform functions essential to life include: Mg, Ca, Mn, Co, Cu, Cr and Zn. Others are extremely toxic (e.g. Cd, Pb and Hg) and homeostatic mechanisms are necessary to control their levels within cells. The life of a living organism relies heavily on appropriate regulation of absorption, intracellular compartment and then translocation of trace metals (Guerinot and Salt, 2001).

Pb is still a potential problem in aquatic systems because of its industrial importance; and comes from ore processing, smelting, and refining operations, motor vehicle exhausts, agricultural runoff and in addition domestic waste water effluents can cause deposition of large quantities of Pb.

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Heavy metal have a potential threat to organisms which attribute to high toxicity as well as aquatic organisms including farmed fish have the ability to accumulate these metals in tissues by directly from the ambient water or by ingestion of food and then become potentially toxic when this accumulation increases to a considerably high level (Tsai et al., 2013; Leonard et al., 2014).

Fresh water fish mainly absorb waterborne metals through their gill epithelia; hence, gills are the first target organs of xenobiotics. Once inside the organism, the metal enters the blood circulation to reach other organs and accumulates most significantly in the kidney, followed by liver and gills (Pretto et al., 2011).

Toxicity of these elements is because of its ability to oxidative stress and damage to living tissues in animals and humans furthermore, the accumulation of these elements can cause intensive lesion to mucus tissues, affecting the intestine and skeletal (Sharma et al., 2014). Thus, heavy metals can provoke problem with fish health and pathological conditions of the fish tissues which include various histopathological lesions in kidney, spleen and muscle (Authman et al., 2012).

Lead is considered as a general protoplasmic poison which is cumulative and slow acting. It is used in different industrial processes therefore; its contamination in water may cause serious environmental problems (Ashraf et al., 2012b).

The levels of heavy metal concentrations including lead Pb were sought among gills, liver, and muscles of red tilapia (*Oreochromis* sp.) that were caught from three different production sites of aquaculture in Jelebu, Malaysia (Low et al., 2011). Furthermore the study of Ashraf et al. (2014) analyzed some metallic elements including lead at ten locations from the former tin-mining catchment that showed the heavy metal pollutions in the water samples from tow locations and were more severe than in other sampling sites, especially tin and lead concentration which were extremely high in the total contents and lead presented mostly in the non-residual fractions in surface water which is combined with organic fraction.

Effect of anthropogenic activities on variations of nutrient concentrations and eutrophication at the lake Bera (TasekBera), Peninsular Malaysia was studied by Gharibreza et al. (2013).

High levels of lead were found in the fish of cyperinidae family (*Rasbora elegans Trichogaster trichopterus Oxyeleotris marmorata*) ranged from 0.07 to 1.78 mg/L were greater than Malaysian Food Act permissible levels due to probably contributed to the lake through various sources such as agriculture activities like oil palm and rubber plantations nearby catchment besides mining activities specially in use of chemical fertilization could introduce heavy metals that including lead (Ashraf et al., 2011).

Previous literature about modern and accurate diagnostic methods for analysis of histological changes in gills by using scanning electron microscope techniques with energy dispersive X-ray analysis EDX in the investigation of heavy metals in aquatic organisms in general such as effects of heavy metal lead acetate on the freshwater amphipod *Gammarus pulex* (Kutlu et al., 2002); in addition analysis of histological changes in different teleost fish tissues and target organs, such as gill ultra structurally has been studied in *Oreochromis niloticus* treated with lead (Pb) at 0.005 mg/L (Atta et al., 2012); and sub lethal concentration (Hassanain et al., 2012). As well as, qualitative and quantitatively analysis of the histopathological alteration in gill of chub *Squalius carolitertii*, barbel *Luciobarbus bocagei* and nase *Pseudochondrostoma* sp. (Pereira et al., 2013), but comparatively few discovery have distinct the histopathological changes under exposure to sublethal concentration of Pb in important gill organ. It has been shown that hybrid tilapia are commercially important in Malaysia and nearby countries (Ponzoni et al., 2010) and have the ability to respond and take up pollutants from the environment (Mokhtar et al., 2009).

The purpose of the present study was to evaluate the median lethal concentration (96-h LC50) of lead, to obtain information regarding the alterations of histopathology in the gills of hybrid tilapia *Oreochromis* sp. in acute lead exposure; and using electron microscopy in conjunction with X-ray Microanalysis to determine the metal content of the gill tissues.

2. Materials and methods

2.1. Fish specimen

Red tilapia fish with an average standard length of 7 ± 0.5 cm and an average weight of (7.2 ± 1 g) were collected from a commercial aquaculture facility in Serendah, Selangor 48200 Kuala Lumpur, Malaysia. Upon arrival the fishes were stocked in group of 25 in 50 L in a semi-static glass aquaria (60 L capacity; 60 cm × 35 cm × 40 cm) system containing UV sterilized (EHK-UVC) de-chlorinated tap water with a pH 7.6 ± 0.06, and maintained at a temperature of 26.5 ± 2 °C and water was kept oxygen saturated by aeration at dissolved oxygen 7.0 mg/L. Tilapia fish were acclimatized to laboratory conditions at a photoperiod of 12 h light and 12 h darkness for 14 days with daily feeding (once per day) of a dry commercial food (pellets with 25% of crude protein). Feeding was stopped 24 h before and during the actual experiment. Aquarium water was replaced every 24 h to reduce contamination from metabolic wastes.

2.2. Exposure to Pb ions

Afterwards, fish were transferred to assay aquaria (20 × 20 × 40 cm) 5 L glass aquaria, which provided aeration via air pumps and air stone diffusers. Fish were divided into four groups (each group was at a stocking density of 10 fish /aquarium). Three as exposed groups and the other group served as the control.

Completely dehydrated Analar grade BDH chemical with 99.5% purity of $Pb(NO_3)_2$ was dissolved in double-deionized water to prepare the stock solution (1000 mg L^{-1}) of Pb. This stock solution was diluted to the desired concentrations with local tap water. Test solutions were replaced by fresh ones of the same respective concentration at every 24 h interval until 96 h exposure (APHA et al., 1998). The median lethal concentration (LC_{50}) during 96 h of exposure determined from the probit transformed concentration – response curves (U.S. EPA (2002)) was 11 mg/L. The LC_{50} values of Pb within 24, 48, 72, and 96 h recorded for *Oreochromis* sp. in the present study with 95% confidence limits (Table 1) were determined by preliminary test. The test concentrations chosen were 50% of the 96-h LC50 value from the acute toxicity test (Thophon et al., 2003) which was 5.5 mg/L Pb. The test procedure was a semi static system with continuous aeration for

Table 1 Median lethal concentration (LC_{50}) of lead in hybrid tilapia, *Oreochromis* sp.

Exposure time (hour)	LC_{50} (mg Pb L^{-1})	95% Confidence limit (mg Pb L^{-1})
24	17.71	15.63–20.53
48	14.91	12.45–18.43
72	12.63	10.18–14.42
96	11.05	9.49–13.16

over 96 h. Three replicates were made for test concentration and control. The characteristics of water quality were: temperature 26 ± 2 °C, dissolved oxygen (DO) 7.25–0.4 mg/L, and pH 7.65 ± 0.5. Fish mortalities were observed daily. Three fish from each aquarium were sampled at 24, 48, 72 and 96 h of exposure.

2.3. Histopathological study

Histopathological analysis was conducted on gills from fishes which were exposed to sub-lethal concentration 96 h $LC_{50}/2$ (5.5 ppm) over 96 h. The fish anesthetized in ice cold water and sacrificed by cervical decapitation and then gill filaments treatments uptake and being fixed in neutrally buffered formalin for 48 h, afterward dried out in a graded ethanol series and inlayed in paraffin. Each block of tissue has been cut into serial sections (6 μm thick) and stained with hematoxylin and eosin (H&E) (Triebskorn et al., 2008). Later the tissues were tested for a wide range of histopathological characteristics and lesions, via general measures of morphology and health alterations.

After examining the tissues the digital images were obtained by using a light microscope Nikon type Eclipse E200, equipped with a Dino eye camera Ø30 mm, employing 10×, 20× and 40× objectives.

2.4. Scanning electron microscopy examination

For SEM, Fish from the experimental and control groups ($n = 3$) were anesthetized in ice cold water and sacrificed by cervical decapitation. The gill filament treatments were fixed at 4-C in phosphate-buffered 8% glutaraldehyde (at pH 7.2) for 1 h. and then post-fixed in 4% osmium tetroxide OsO_4 in the same buffer overnight to increase electron density. Tissues were dehydrated in ascending series of ethanol concentrations and then dehydrated in a grade series of ethanol acetone mixture solutions (Pandey et al., 2008). Afterward, they were dried in a critical point-drying apparatus (CPD 030, LEICA EM) with liquid CO_2 for 30–60 min, specimens were mounted onto aluminum stubs and coated with gold by a coating machine (SCD005 Sputter Coater, LEICA EM). Morphological analysis was undertaken using a JEOL JSM-7001F, Japan Scanning Electron Microscope at an accelerating voltage of 15 kV.

2.5. Energy dispersive X-ray analysis (EDX)

The percentage of the weight of the mineral contents through the cross-section of gills was quantified by energy dispersive X-ray (EDX) spectroscopy analysis using a scanning electron microscope (JEOL JSM-7001F, Japan) equipped with EDX (OXFORD Instrument X-Max). All the specimens were analyzed under the same conditions in order to minimize matrix effects. Data were collected at a 15 kV accelerating voltage with a 10 l A operating current and a 15 cm working distance. For the comparison with results obtained from the SEM image analysis, the same samples were used for the EDX analysis.

3. Results and discussion

In the present study the untreated gills showed bearing of four pairs of gill lamellae and both sides were supported by bony structure of gill arch. The characteristic arrangement of primary and secondary lamellae is demonstrated in Fig. 1(A1 and B1). The secondary lamellae showed numerous channels of blood capillaries each separated by single layer pillar cells; chloride cells and mucous cells were located (Fig. 1C1). The gills after 72 and 96 h Pb exposed fishes show proliferation and hypertrophy of epithelial cells occasionally resulting in fusion in adjacent secondary lamellae (Fig. 1A2). At the tips of the secondary lamellae bulb shape of the large pavement cells at 72 h was found (Fig. 1B2) and an increase in chloride cell density at 96 h exposure was observed (Fig. 1C2).

These results concur with the study of Triebskorn et al. (2008) which showed increased large mucocytes at the tips of the secondary gill lamellae, cellular necrosis, cellular hypertrophy in *Leuciscus cephalus* exposed to heavy metals including lead element.

The level of accumulation in distinct organs depends on uptake and elimination rates which are different from one tissue type to other; subsequently, metal accumulation in fish has produced damage to gill structure (Giari et al., 2007).

Gills are sensitive subjects for identifying under the effect of heavy metals on it by various histopathological alterations including hypertrophy and hyperplasia of epithelial cells, lamellar fusion, hyper secretion of mucous, and lamellar aneurysm (da Silva et al., 2012; dos Santos et al., 2012; Pereira et al., 2013).

Scanning electron microscopic images established the results observed by light microscopy at low resolutions. In control fish, there are four gill arches of gill on each side of the body. Every one supports numerous gill filaments which are arranged in two rows called hemi branches, arrangements of primary and secondary lamellae were organized with consistent interlamellar space.

Pavement cells (PCs) were found more abundant in the filament epithelium, while chloride cells and mucous cells were comparatively limited and located mainly at the bases of lamellae and on the trailing edge of the filament. Furthermore, high resolution microscopy demonstrated characteristic surface model of PCs shaped by long microridges and other concave apical surfaces of the CCs that were covered by microvilli (Fig. 1A–D).

X-ray microanalysis with energy dispersive spectroscopy was used to scan primary and secondary lamellae of gills to determine their metal composition. Eight elements were predetermined for analysis: Ca, P, Cd, Cu, Mg, Hg, Pb and Zn and their abundance was recorded as raw X-ray counts.

The EDX analysis of the normal gills of red tilapia fish has indicated that there are five elements viz. Calcium, Phosphorous, Magnesium, Copper, and Zinc present in the

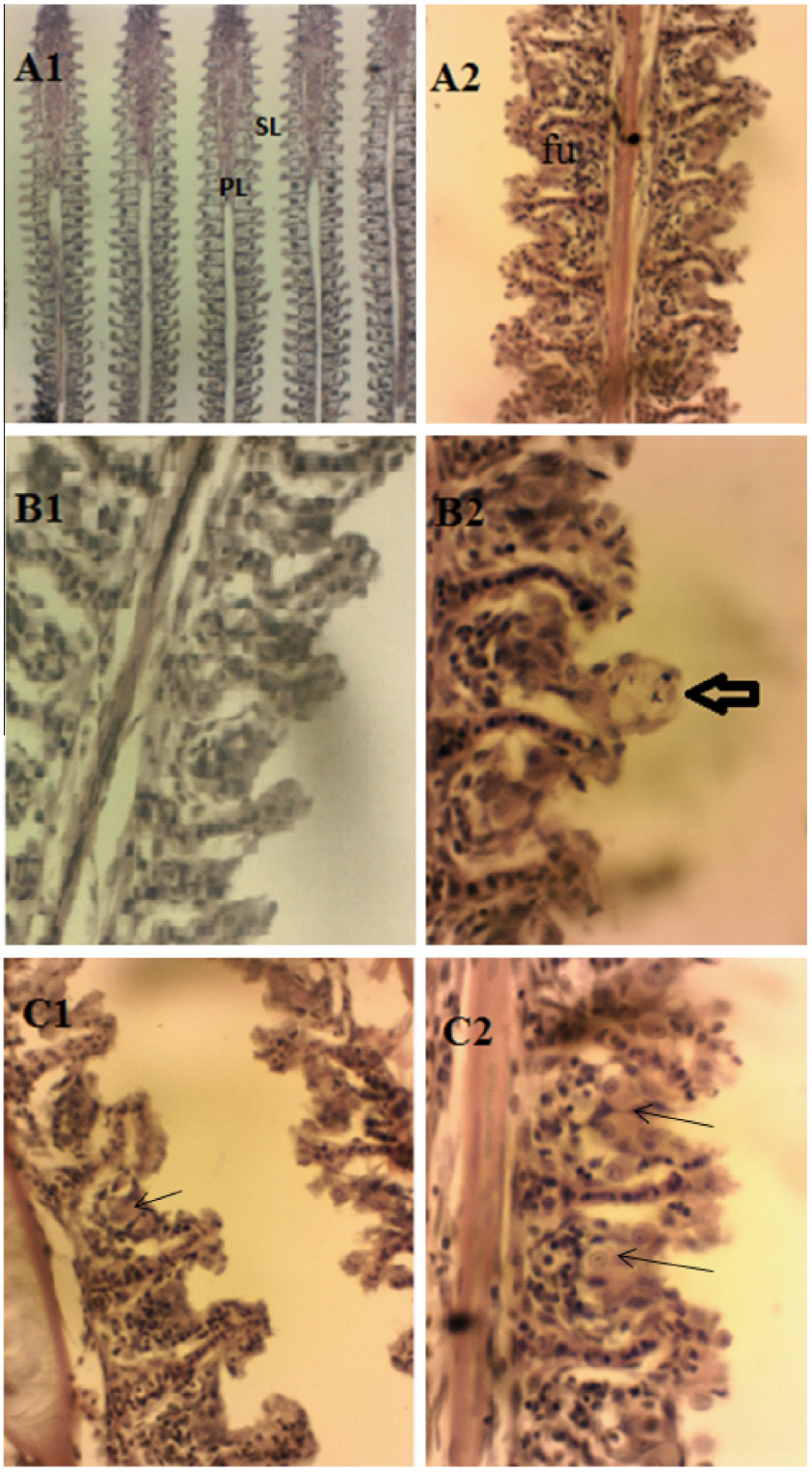

Figure 1 Light microscope microphotographs of the gill filaments of *Oreochromis* sp. in the control (A1, B1, and C1) and experiment (A2, B2, and C2). (A1) General view demonstrates the characteristic arrangement of primary (PL) and secondary lamellae (SL) in gills of control fish.A2 shows fusion of adjacent secondary lamellae (fu) in Pb exposed fish at 72 h (×200). (B2) Large pavement cells at the tips of the secondary gill lamellae at 72 h (Arrow). C2 shows presence an increase in chloride cell density (←) (×400) (H&E).

gills. Among these elements, Ca and P have the maximum percentage while Hg, Cd, Pb were not detected (Fig. 2).

Heavy metal pollution of the aquatic environment is a subject of considerable concern. These metals tend to accumulate in organisms and have been found to have a variety of adverse effects on fishes. Higher concentrations of lead, cadmium and mercury were toxic to the fishes; even lower concentrations considered toxic to the fishes (Atta et al., 2012) (see Fig. 3).

SEM of gills from treated red tilapia with Pb ions presented in the present study revealed impairment and disturbance of bony ossification of gill filament and lamellae and also have been shown abnormalities and changes in architectural formality in gill filaments (Fig. 4C) and coagulate necrosis in pavements cells with disappearance of its architecture to gather with its microridges because of the influence of the toxicity of lead ions which produced changes in the ultrastructure and chemical composition of gill filaments (Fig. 4B); this is described by review of Jezierska et al. (2009) about disturbance by heavy metals on early development of fish may be cause by metal toxicity which reduces gill calcium uptake and resulting in changes in gill filament properties and then they become flexible.

In addition the presence of lobulated areas with the deep dark inter lamellar space of gills may be due to the mixing of increased mucus secretion with inflammatory fluid (Fig. 4C).

This is similar to the study of Hassanain et al. (2012) which explained the Pb element analysis via SEM with EDX technique on gills of Nile fish *Oreochromis nilotica* which have been treated by the lead acetate (14.6 mg/L); their results indicated that gill filament and pavement cells have distinct degeneration; as well as, revealed impairment and disturbance of bony ossification of gill lamellae and filaments due to bony proliferation changes, and also the pavement cells showed coagulated necrosis with the disappearance of its architecture and microridges which may be attributed to the metal toxicity which reduces gill calcium uptake and resulting in changes in gill filament properties. Also observed was the deep dark inters lamellar space with lobulated areas that may be due to the organization of the increased mucous mixed with the inflammatory fluid.

Lead exerts its effect, physiologically and biochemically as a mimetic agent substituting for essential elements participating in metabolism such as calcium, iron and zinc. Specially, it directly interferes with zinc and iron in the biosynthesis of heme, in the function of sulfhydryl group rich protein enzymes and in protein synthesis in general either directly or indirectly. In addition lead binds to different kinds of transport proteins including, metallothionein, transferrin, calmodulin and calcium-ATPase resulting in the loss of metabolic function which continues to be a primary hypothesis underlying the detrimental effects of lead exposure (Corpas et al., 2002; Lewis and Cohen, 2004; Zeitoun and Mehana, 2014).

The gill is an organ that has a high degree of specialization and vital functions. One of these functions as the area in which where gas exchange occurs in breathing; as well as working as a site for the removal of waste products of nitrogenous metabolism in addition to mineral balances and preservation of acid-base. Working to solve the osmotic problem compared to the surrounding medium by contributing to sustain the high osmotic pressure in the extracellular fluids of fish that live in hypotonic environment. This is due to the possibility of absorption of active ion via the gills and by prevent and hinder the loss of the ion through the membranes and water flow to their tissues. Thus, heavy metals affect both oxygen absorption and osmoregulation in fish. Subsequently it will suffer from numerous histopathological changes in gill (Lehtinen and Klingstedt, 1983).

An examination by EDX in scanning electron microscope showed the spectrum increasing in the lead element percentage and this is evidence of a cumulative susceptibility to the Pb metal in the fish gill of *Oreochromis* sp. and at the same time this metal effected on to reduce the calcium and phosphorus as in Fig. 5.

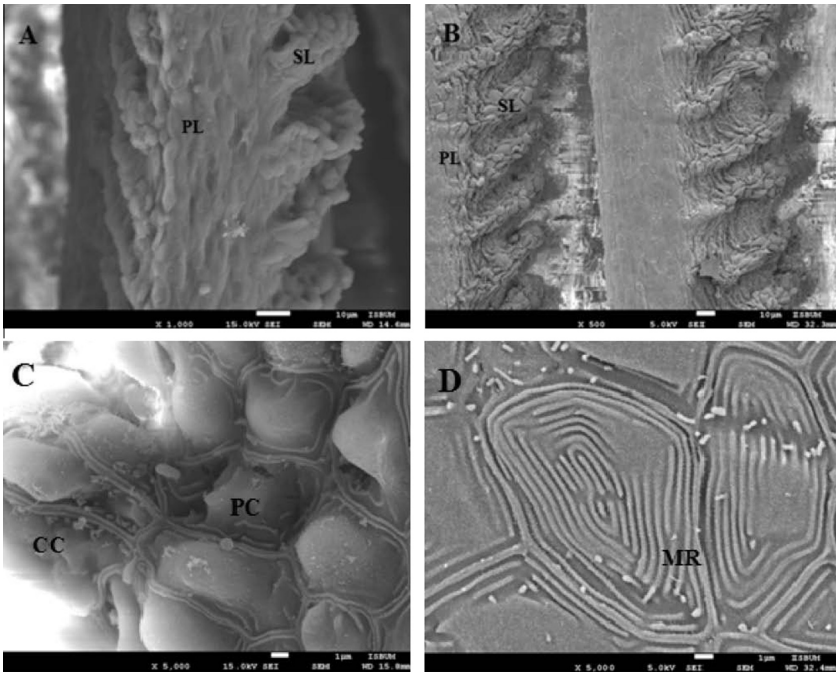

Figure 2 Scanning electron microscopic micrographs of gills *Oreochromis* sp. (A) General view of control fish gill filaments and lamellae showing normal morphological features. (B) Primary lamellae (PL) and secondary lamellae (SL). (C) Note well-organized pavement cells (PC), chloride cells (CC) in base edge of secondary lamellae. (D) organized microridges in pavement cells (MR).

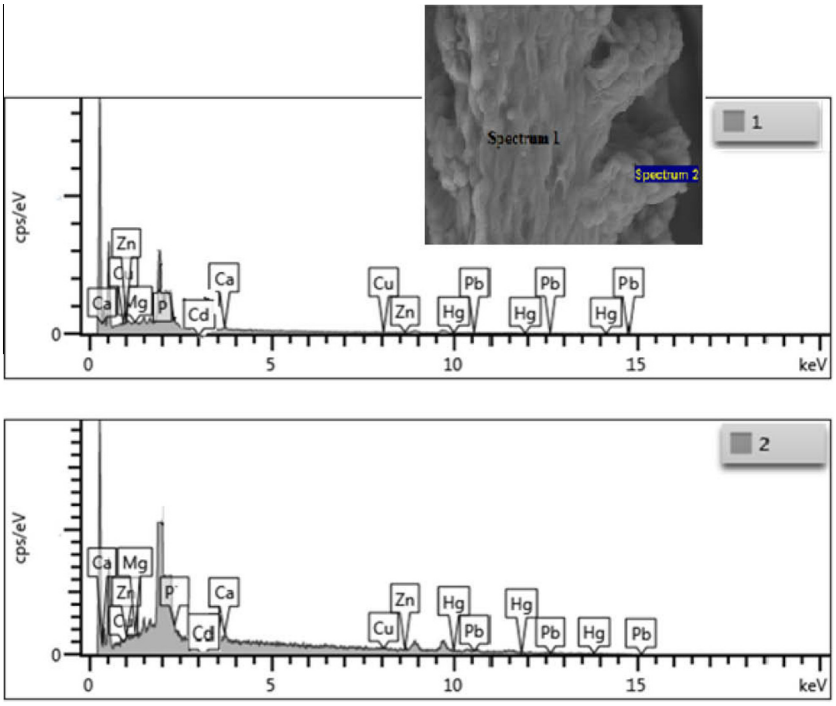

Figure 3 Scanning electronic micrograph and energy dispersive X-ray spectroscopy microanalysis of the control gill tissue. X-ray spectrum shows only essential elements usually present in biological specimens Ca, P, Cu, Zn, and Mg and not detecting of Cd, Hg, Pb in primary lamellae area (1) and secondary lamellae area (2).

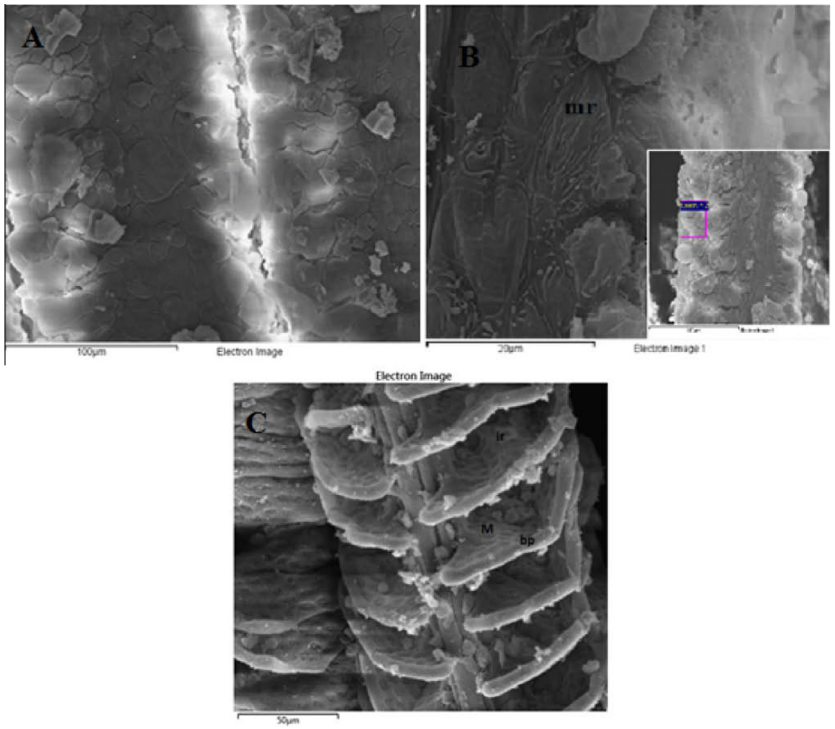

Figure 4 Scanning electron micrographic image of gill from *Oreochromis* sp. at 96 h exposure to Pb ions (A) showing fusion of secondary lamellae and loss of normal architecture and increased severity of morphological changes. (B) Demonstrating disappearance of microridges in pavement cells at 96 h exposure to Pb ions. (C) Showing the presence of mucus secretion (M) in interlamellar region (ir) and bony projection (bp) that appeared on the lamellae surface.

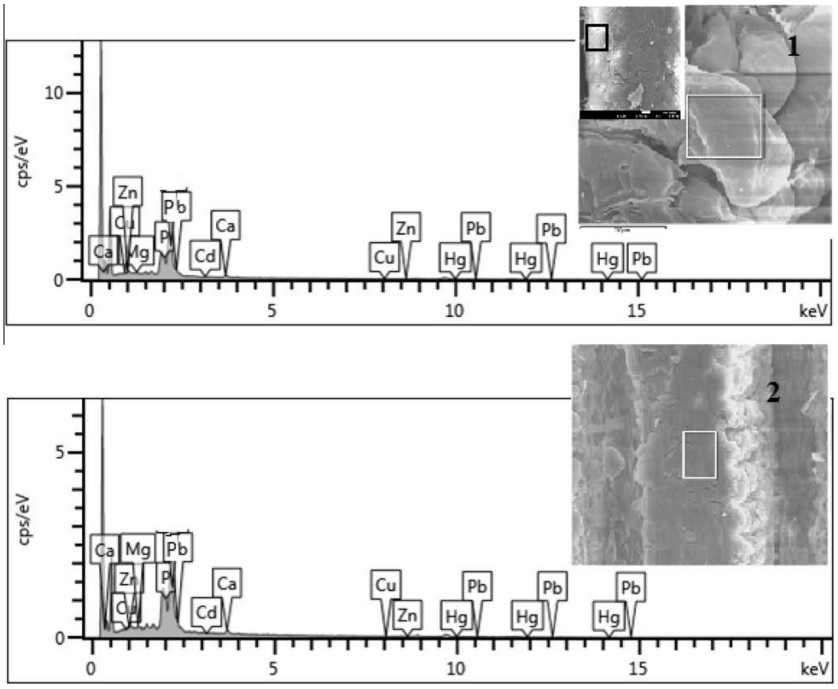

Figure 5 SEM and EDX microanalysis of the gill lamellae from *Oreochromis* sp. after 96 h exposure to Pb ions. Elemental analysis spectrum shows appearance of Pb in weight percentage (25.27%) in secondary lamellae area (1) and (27.47%) in primary lamellae (2) with low peaks of essential elements (Ca, P, Zn, Cu and Mg) and not detecting of Cd and Hg.

The presence of lead in exposed fish gills in high concentration is due to that Pb ion from waterborne binds with the mucus layer which exists on general body surface and particularly on gills of the fish (Tao et al., 2000). Adding to that, lead has an affinity with fish biomass which is considered a potential biomass to remove Pb^{+2} ions from synthetic solutions with lead contaminated water (Ashraf et al., 2012).

Anyway, concentrations of metals in the tissues of fish gill reflect the presence of these concentrations in the ambient water, whereas increasing concentrations in the liver indicate the metal storage in longer period (Rao and Padmaja, 2000).

In the present study lead had the impact on the histopathological changes in gill filaments including necrosis, fusion and proliferation in epithelial cells; this is consistent with Olojo et al. (2005) about study of cat fish *Clarias gariepinus* which exposed to environmental pollution such as lead noticed breakdown of pillar cell system then resulting in capillary congestion.

Also corresponds with study of Vasanthi et al., 2013 found the accumulation of heavy metals including lead, iron and zinc was high concentrations in the gill tissues due to the mechanism of the body's defense and this organ is the main way for the entry of pollutants from the water; that resulting in several histological lesions observed such as slight malformation of the gill lamellae in addition fusion of adjacent lamellae were more obvious and more prevalent in the fish *Mugil cephalus* which was found in polluted environment; and this alteration could be a protective effect for minimization the quantity of surface area in susceptible gill .

Heavy metals have effects on the regenerations or degenerations of the cells which were recorded by Atta et al. (2012) who found that the cytoplasm of the cells is vacuolated with multi-nucleoli in treated fishes *Oreochromis niloticus* with Pb at 0.025 mg/L; and this may lead to cell proliferation irregularly.

The present study provides additional understanding into damage of lead on fish histopathology. Ultrastructural response in the tissues of gills already confirms the high sensitivity and demonstrates the early toxic effect pertinent to the environment. These obtained results demonstrated that exposure to lead can cause possible deleterious consequences for both the survival and health status of fish; Dysfunction in the gills may lead to impairment and an imbalance in the gas and ion exchanges. However, there is a need for more additional experiments to enhance understanding of toxicity mechanisms for this element.

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