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Cytopathological changes of gills and muscles in Asian sea bass, *Lates calcarifer* (Bloch) exposed to mercury

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Abstract
Heavy metal concentrations of mercury and the associated structural deformities in the gills and muscles of Asian sea bass, *Lates calcarifer* were observed for sublethal toxicity studies. Cytopathological alterations in gill tissues displayed oedema of epithelial cells, ballooning dilation, partial epithelial lifting, and damaged pillar cells; the muscle displayed shortening of muscle bundles, oedema and necrosis, atrophy of muscle bundles, and vacuolar degeneration in muscle bundles; The present study demonstrated that all the treated body tissues exhibited significant damage with response; among the body organs, the liver and brain are important target organs for mercury toxicity in *L. calcarifer*, which suggest that biomarkers are useful in assessing the model organism for toxicity studies.

Keywords: *Lates calcarifer*, mercury, cytopathology

Introduction
Mercury has no known metabolic functions in human beings and therefore even low concentrations in the body may be measured to be potentially harmful. Mercury in fish and seafood occurs mainly as methyl mercury and partly as inorganic mercury bound to organic molecules. Mercury compounds exert their action by altering the membrane structure, and thus seriously affect the permeability character of cell types. The inability of mercury - exposed fish to maintain its ionic balance could be attributed either to a decreased uptake of ions via gills or to an increased loss of ions via gills or kidney. Histopathology refers to the microscopic examination of tissues in order to study the manifestations of disease or damage. Toxicological histopathology gives useful data regarding changes induced by chemicals in pesticides at the tissue and cellular levels. All tissues and organs in the body of an animal are considered potential targets for the toxic effects of any chemical compound, for example pesticides. A histopathological assessment clarifies the nature of tissue alteration and the extent of damage indicating the toxic nature of the compound. The advantages of using cytopathological biomarkers in environmental monitoring are manifold. Biomarkers allow examining specific target organs, such as gills, kidney and liver, responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish [1, 2, 3]. Furthermore, the alterations found in these organs are generally easier to identify than functional ones and they serve as warning signs of damage to animal health [4].
Changes in fish gill reflect the most commonly recognized responses to environmental pollutants [5]. Also, gills are the first target of waterborne pollutants because they are the main place for mercury uptake; they also have a constant and direct contact with the outdoor environment. Monitorisation of histological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in field and experimental studies. Numerous types of gill damage have been documented in fish exposed to toxicants or in populations sampled from polluted environments [6, 7 & 8]. Most of the gill histo-pathological changes are largely non-specific as deep-rooted by the occurrence of similar alterations under a wide range of toxicant-exposure conditions [9]. The important functions of fish gills are respiration, acid-base balance, excretion, and osmoregulation and they are one of the most important organs for the uptake of inorganic mercury in fish [10] which also become efficient indicators of water quality [11, 12]. Liver is allied with detoxification and biotransformation due to its position, function and blood and it is one of the vulnerable organs damaged by a diversity of toxicants [13]. Heavy metal or pesticide exposure results in degenerative changes in the muscle tissue [14, 15]. Stomach and intestine are important organs of digestion and absorption exposed to heavy metal pollutants. Histological alterations have been reported in the intestine of fish as a result of exposure to different toxicants [16, 17, 18, 19]. In this paper we report the cytopathological examination of gill, muscle, intestine, brain and liver tissues of Asian sea bass, *Lates calcarifer*.

Materials and Methods
Experimental animal collection and maintenance
The experimental animal *L.calcarifer* were collected from Rajiv Gandhi Centre for Aquaculture (Tamil Nadu, India) and acclimated to laboratory conditions for 15 days. During this period, fishes were maintained in 100-L capacity aquaria with water and equipped with filter and oxygenation systems. During acclimation period, salinity (15±2 ‰), density (1.021–1.031 g/cm3), temperature (25 – 26 ˚C) and nitrite and nitrate concentrations were measured and kept constant (dissolved oxygen 6.5±0.7 mg/L; hardness 100 mg $CaCO_3$/L and the absence of heavy metals). For the experimental duration, the animals were maintained under a natural light/dark cycle and fed every second day with commercial fish food. They were starved for 24 h before and during the experiment.

Chemicals used
For preparation of stock solution, 1.368 g of Mercuric Chloride (Hg Cl_2) (Merck) was dissolved in l litre of double-distilled water and used as stock solution. It was stored in a clean standard flask at room temperature in the laboratory.

Experimental procedure
Test concentration
Fish were exposed to nominal 0.127 and 0.254 ppm of mercury. Doses were theoretically sublethal, 10% and 20%, respectively, of the maximum acceptable toxicant concentration (MATC), which was 1.27 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 96h LC50 (AF= MATC/LC50 = (NOEC–LOEC)/LC50).

System design
A recirculation closed system was set up according to Muthuvan [20]. The experiment was carried out in 300 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 acclimatisation in a holding tank, ten healthy fish (7.17 ± 0.27cm in length and 9.18 ± 0.47gm 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 [21]; water quality (dissolved oxygen, temperature, pH and salinity) was measured Every day and water chemistry (ammonia nitrogen, nitrite nitrogen, nitrate nitrogen) was measured twice weekly. All chemical parameters were determined following the techniques of APHA [21] 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 Every day for each concentration. Two fishes from each aquarium were sampled at 0, 7 and 28 days post-exposure.

Table 1

Parameters	Range	Mean+S.D
Dissolved oxygen (mg/l)	6.5–7.5	6.97±0.15
Temperature (ºC)	25.9–28.7	27.1±1.25
Salinity (%)	26.4–32.2	29.0±1.87
pH	6.51–8.74	7.50±0.91
Ammonia nitrogen (mg/l)	0.02–0.76	0.47±0.31
Nitrite nitrogen (mg/l)	0.03–0. 73	0.64±0.51
Nitrate nitrogen (mg/l)	0.59–0.89	0.71±0.12
Actual mercury concentration (ng/l^{-1})	4.4 -5.2	0.017±0.02

Cytological analysis
The gills and muscle were fixed in 10% buffered formalin for 24 h, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (5 mm thick) were stained with haematoxylin–eosin. The thin sections of the tissues were stained by haematoxylin and eosin for observation by the Nikon bright field transmission microscope with Koehler illumination and automatic exposure unit was used.

Results
Cytology of Gills
Cytological study of the gills shows a distinctive structural association of the lamellae in the untreated fish (Plate 1 A). There are four gill arches on each side of the buccal cavity and each arch is composed of numerous gill filaments. The primary gill lamellae are flat leaf - like structures with a central rod-like supporting axis and a row of secondary gill lamellae on each side of it. The secondary gill lamellae (SGL) were equally spaced along the columnar structures with intact cellular layer attached at their bases with the primary gill lamellae (PGL) and free at their distal ends. Secondary gill lamellae were composed cells, which were contractile and separated the capillary channels. The normal secondary lamellar epithelium was simple, consisting of a thin single or double sheet of epithelial cells, blood vessels and a row of pilaster cells. The region between the two adjacent secondary gill lamellae is known as interlamellar region. The lamellae are lined by a squamous epithelium composed by pavement and no differentiated cells. One to two erythrocytes were frequently observed within each capillary lumen. Chloride cells are seen as large epithelial cells with light cytoplasm, usually present at the base of lamellae. At the base of the lamellae mucous cells were present in the filamental epithelium and they lacked the light cytoplasm.

Cytopathology of Gills
Lower concentration of mercury treatment at 7 days resulted in numerous forms of histopathological alterations in the gill filaments including lifting of lamellar epithelium (LLP) and hyperplasia (HP) in the distal region (Plate 1 B). Changes were observed as proliferation of filamentary epithelium (PFL), hyperplasia of the epithelial cells (HPER), and epithelial lifting (EL) in primary lamellae at lower concentration of mercury treatment in 28 days (Plate 1C). The separation of secondary gill lamellae (SSGL), and the separation of epithelial cells from pillar cells (SEPC) were noticed. The fusion was of the highest order and no distinction could be made among pillar cells, epithelial cells rupture, vaculations, blood cells and mucus cells in such region were observed in higher concentration at 7 days of mercury exposure (Plate 1D). After 28 days photomicrograph of gill treated with higher concentration of mercury showed cytoarchitectural distortion of the lamella with overlapping of the primary and secondary lamella. Considerable mucous and granulated eosinophilic cells were witnessed in their cytoplasm. Extensive vacuolisation was noticed with prominent disruption of the epithelium and the blood cells accumulated in this spherical structure. The infused secondary lamellae were thinner compared to their controls. The epithelial cells were seen between the fused secondary lamellae. Fusion of the Boundary of the secondary lamellae (FSGL) increased with exposure periods

(Plate 2E). Other histopathological changes were cellular hypertrophy or hyperplasia (HP) in the epithelial layer of primary filaments and fusion of secondary lamellae. Other observations during the experimental period include epithelial rupture (ER) interstitial oedema and blood congestion in the vascular axis of primary filaments. In addition, a few telangiectasis were also observed at gill lamellae (Plate 2E). Oedema of epithelial cells, ballooning dilation, partial epithelial lifting, damaged pillar cells, enlargement of primary lamellar epithelium, aneurism of secondary lamellae, congestion of blood spaces, infiltration of the secondary lamellae (SSGL) cellular hyperplasia, mucin filled space, damaged gill rackers, fusion of secondary lamellae, infiltration of erythrocytes, degeneration of lamellar epithelium (DGL) breakage of gill rackers, necrosis of lamellae, blood congestion and hyperplasia of epithelium were also detected (Plate 1 F). The main response of gill epithelium was reduction in permeability.

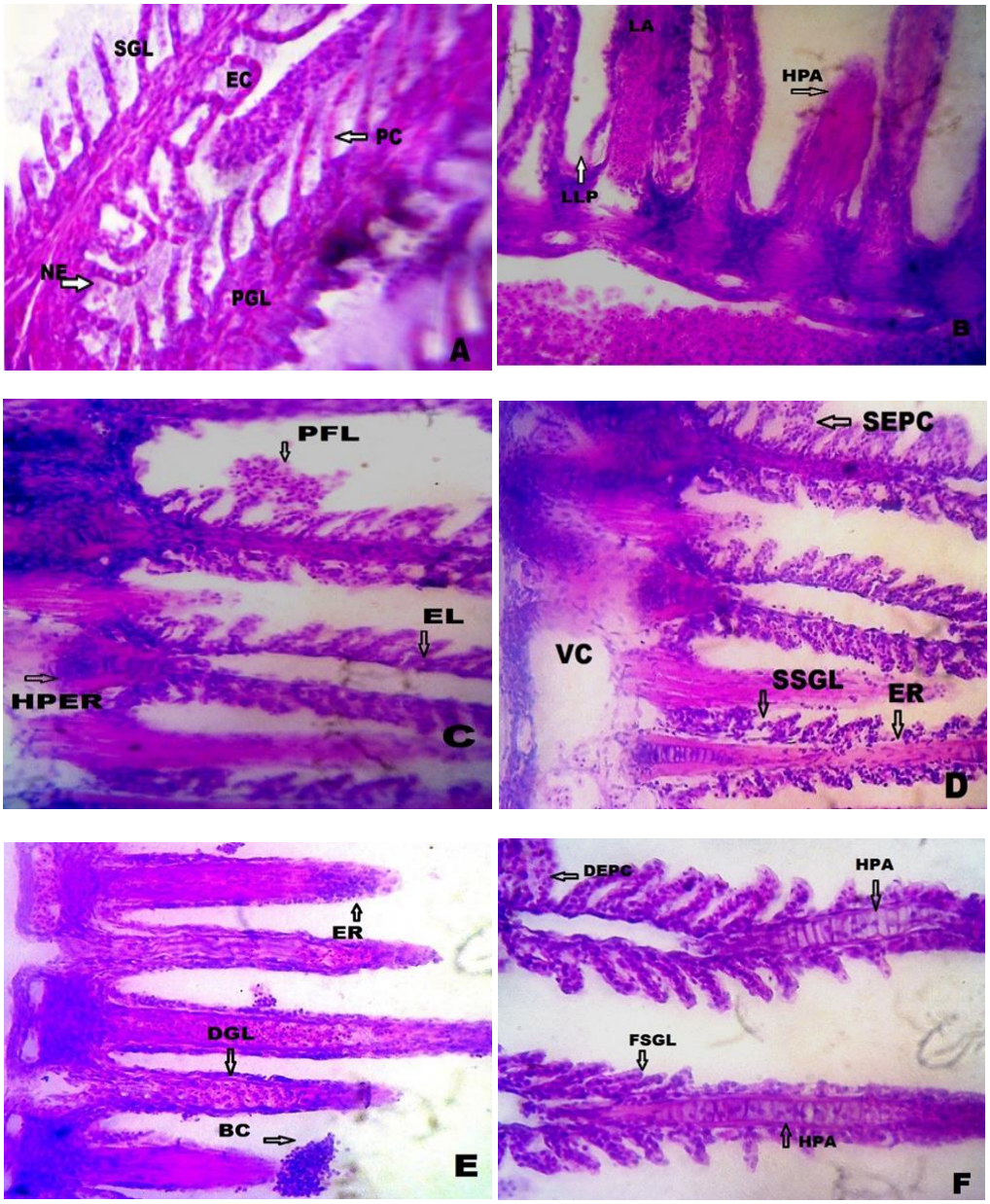

Fig 1: Cytological changes of gills in *L.calcarifer* Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A: Control B:After 7 days of exposure to 0.127 ppm concentration of mercury, C: After 28 days of exposure to 0.127 ppm concentration of mercury, D: After 7 days of exposure to 0.254 ppm concentration of mercury, E & F- After 28 days of exposure to 0.254 ppm concentration of mercury

Abbreviations Used: PGL - Primary gill lamellae, SGL- Secondary gill lamellae**,** PC- Pillar cells**,** NE- Nucleated erythrocyte**,** EC- Epithelial cells**,** HPA- Hyperplasia**,** LA- Lamellar aneurysm**,** LLP - Lifting of lamellar epithelium**,** PFL- Proliferation of filamentary epithelium**,** EL- Epithelial lifting**,** HPER- Hyperplasia of the epithelial cells**,** VC- Vaculation**,** SSGL- Separation of secondary gill lamellae**,** ER- Epithelial rupture**,** SEPC - Separation of epithelial cells from pillar cells, DGL- Degenerated gill lamellae. BC- Blood congestion, DEPC- Degeneration epithelial cells, FSGL- Fusion of secondary gill lamellae

Cytology of Muscle

Photomicrograph of the muscle (Plate 2A) depicted the presence of normal myotomes with equally spaced muscle bundles. Muscles are composed of segmental myomeres. Each myomere is regarded as apparent muscle and its fibres are parallel to the long axis of the body. This muscular layer is covered with skin which is formed of three layers (epidermis, dermis and hypodermis). Also, the skin is covered with an epithelial layer. The control muscle bundles were intact with signs of high metabolic activity. The skeletal muscles of fish are highly active helping in their navigation through water. Muscle bundle, muscle fibre, nuclei and endomysium are distinct. Histological study of muscle tissues of the control sea bass showed various muscle layers i.e. epidermis, dermis, myo-epithelium and normal myotomes with equally spaced muscle bundles which indicated the fish to be in unstressed conditions (Plate 2 A).

Cytopathology of Muscle

In contrast, muscle tissue of experimental sea bass at 7 days exhibited lower concentrations of mercury exposure with prominent changes like disintegrated myofibrils (DMF), and intermyofibrillar space (IMS) (Plate 2B). Granular aggregates (GA), Myofibrils of endomysium detachment (MED), and severe intramuscular oedema (SIO) were also observed in lower concentrations at 28 days (Plate 2C & D). The muscle of fish exposed to higher concentrations of mercury at 7 days showed detachment of muscle fibre (DMF), vacuolar degeneration in muscle bundles, spitting of muscle fibre, focal area of necrosis shortening of muscle bundles (SMB), necrosis of muscle bundles, thickening of muscle bundles (TMB) and a number of other histological changes (Plate 2 E). Exposure to sublethal concentrations of mercury at higher concentration in 28 days marked thickening and separation of muscle bundles, haemolysis, necrosis, lesions with reduced compactness and pronounced intramuscular oedema with minor dystrophic changes. Splitting of muscle fibres and vacuolar degeneration in muscle bundles were considered to be significant histopathological changes (Plate 2F).

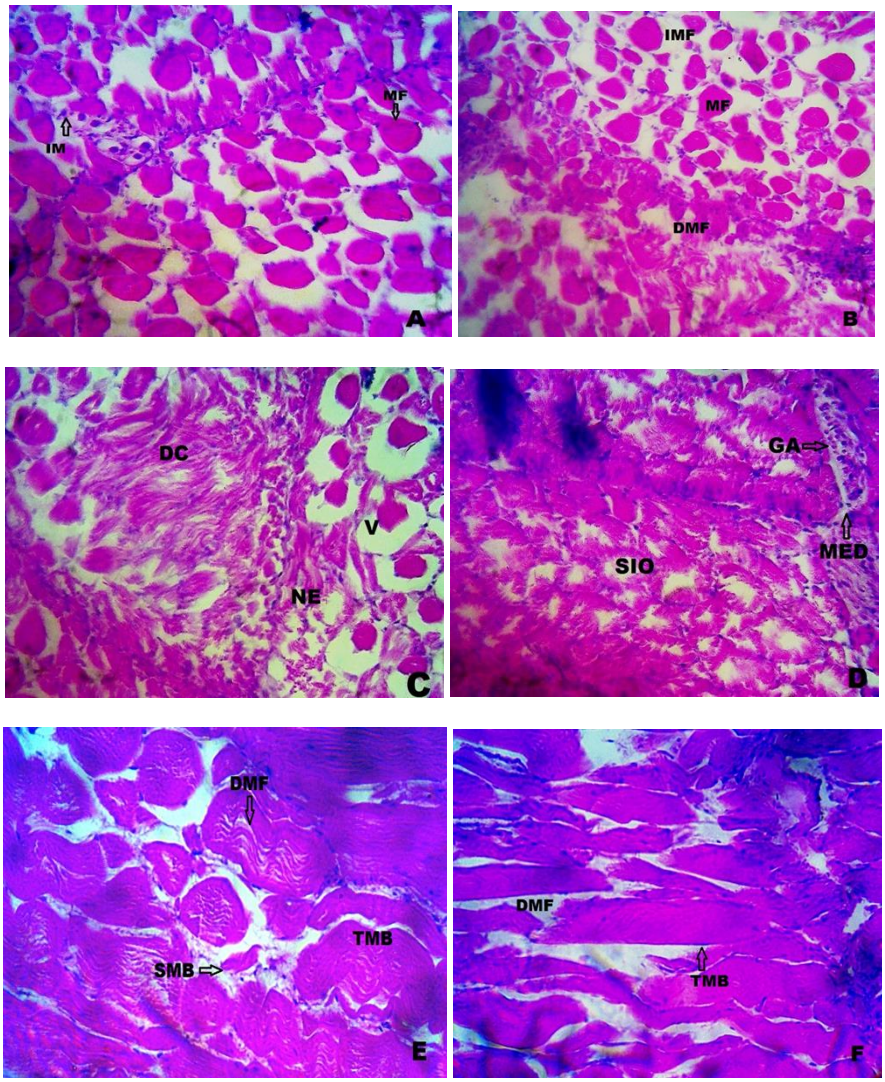

Fig 2: Cytological changes of muscle in *L.calcarifer*. Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A: Control, B: After 7 days of exposure to 0.127 ppm concentration of mercury**,** C & D- After 28 days of exposure to 0.127 ppm concentration of mercury, E- After 7 days of exposure to 0.254 ppm concentration of mercury**,** F- After 28 days of exposure to 0.254 ppm concentration of mercury.

Abbreviations used: IM- Intestitial materials, MF- Muscle fibre, IMF- Inter myofibrillar space, DMF -Disintegrated myofibrils, DC - Dystrophic changes, V- Vacuole. NE- Necrosis, GA- Grannular aggregates, SIO- Severe intramuscular oedema, MED- Myofibrils to endomysium

detachment, SMB- Shortening of muscle bundle, TMB- Thickening of muscle bundle.

Discussion

Cytopathology is the microscopic study of a diseased or a damaged tissue; it is an important tool of anatomical pathology since accurate diagnosis of diseases usually requires cytopathological examination of samples. Biochemical studies may give an idea of the pathological state of the animal, yet a clear picture of cytoarchitectural changes produced during chemical intoxication can be traced clearly only by cytopathological studies. These studies help assess the extent of pollution in the ecosystem caused by pollutants such as pesticides, and offer an exceptional opportunity to detect the effect of pollutants in various organs and organ systems of any organisms.

The gills are important organs for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion [22]. Gills are efficient tools for biomonitoring potential impacts because of their contact with water and high permeability [23]. The damage caused to gills in Spiny lobater, *P. homarus* and Asian sea bass, *L.calcarifer* could be a direct result of the heavy metal in copper which entered in water [24, 25]. The gills are among the most vulnerable organs of the teleosts because of their extrinsic location and cherished contact with water. Therefore, they are accountable to damage by irritant materials dissolved or suspended in water [26]. It is assumed that during the process of gill uptake, metals are adsorbed onto sites in cell walls and cell membranes [27]. Karlsson *et al*., [28] mentioned that, the increase in cellular layers of lamellar epithelium may be due to an increase in the number of mitotic divisions of the lamellar epithelium. Kantham *et al*., [29] suggested that the gill hyperplasia may increase epithelial thickness to retard into the blood stream. Cell proliferation with thickening of gill filament epithelium may lead to lamellar fusion. The fusion and hyperplasia of gill lamellae may be induced by the effect of the toxin altering glycoprotein in the mucus covering of cells, thus affecting the negative charge of the epithelium and favouring adhesion to the adjacent lamellae [30]. Mallatt [9] reported that excessive secretion of mucus, lifting of the gill epithelium and fusion of the gill lamellae were all protective mechanisms that reduce impacts of pesticides on the gill tissue. Oedema with lifting of lamellar epithelium could serve as a mechanism of defence, because separation of lamellar epithelium increases the distance, which waterborne pollutants must diffuse to reach the bloodstream [31]. 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 an increase in the distance between the external environment and the blood, they serve as a barrier to the entrance of contaminants. The increased distance between water and blood, impairs oxygen uptake [32]. These cytopathological changes of the gills likely resulted in hypoxia, respiratory failure problems with ionic and acid-base balance [5]. In addition, the pathological changes in the chloride cells may indicate osmoregulatory dysfunction, which is the main function of the chloride cells [33].

Histological study of muscle tissues of the control fish showed various layers i.e. epidermis, dermis, myo-epithelium and normal myotomes with equally spaced muscle bundles, which indicated the fish to be in unstressed conditions. In contrast, muscle tissues of experimental sea bass exhibited prominent changes such as shortening of muscle bundles, oedema and necrosis (Fig. 2C, 2D). Elongation of muscle bundles was also observed. Although, muscle is the most edible part of fish body, it is also the tissue which is in close contact with pollutants dissolved in water [34, 35]. According to Saad *et al*., [36] if fish inhabiting polluted water displayed epithelial lesions in muscle tissues then that would most probably be invaded by microorganisms causing severe epidermal pathology, resulting in degeneration of muscle bundles. The results of the present study on market carp are corroborated by the findings of [37], who noted several histological variations such as destruction and vacuolation in the muscle cells of *Oreochromis* species, following exposure to chromium. Patnaik *et al*., [38] studied similarly, the histology of *C. carpio* exposed to sub-lethal concentrations of lead and cadmium. The authors reported marked thickening and separation of muscle bundles with intracellular oedema. Similarly, degeneration of muscle bundles along with aggregation of inflammatory cells between them, focal areas of necrosis, vacuolar degeneration in muscle bundles and atrophy of muscle bundles have been reported in fish exposed to different pollutants [39].

Conlusion

Most studies on mercury toxicity in aquatic organisms have considered the exposure media in accordance with the most abundant form/species of mercury found in the uptake pathway. Therefore, effects of exposure to inorganic mercury via water have been neglected in the past studies, even though it can be accumulated after ingestion. Results of the present research show that the effect of mercury concentrations were tested in various tissues using the cytological techniques in fish. This indicates higher susceptibility of larvae to mercury exposure which could increase vulnerability to predation and therefore, endanger fish populations in contaminated environments. Changes in juvenile fish biomarkers were observed and were dependant on the tissue type and period of exposure. Hence this study can be used as a tool for creating awareness among the local farmers and compare the sensitivity of various species of aquatic animals and potency of effluent using LC_{50} values and to derive safe concentration so that the use of the highly toxic heavy metal can be minimized.

Conflict of Interest

The authors declare that they have no conflict of interest

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