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Research Article

Histopathological and oxidative impairment in liver and gills of *Catla catla* exposed to artificial seawater

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ABSTRACT

The aim of the present investigation is to reveal the antioxidant enzymes response and histopathological alterations in the liver and gill tissues of *Catla catla* fingerlings to different concentrations of artificial sea water (ASW). LC_{50} values for the response towards the tolerance for ASW at different time intervals; 24h, 48h, 72h, and 96h and at different concentrations were drawn through the percent mortality determination. The estimation of percent mortality confirmed the fingerlings could tolerate the concentration of ASW up to 47.5 %. Lipid peroxidation assay revealed that the concentration of ASW is directly proportional to the LP levels. The analysis of the antioxidant enzymes (CAT, SOD, GPx & GR) was concentration dependent. CAT & SOD showed an increase while GPx & GR showed a decrease with the increase in concentration of ASW. The reported damage was confirmed by the histopathological analysis that revealed the alterations in gills and liver. The extent of damage to the tissues was proportionate with increased concentration of ASW. The present study clearly demonstrated that Liver and Gill tissues exhibited significant damage with response for artificial sea water toxicity in this species could be possibly used as a model organism for toxicity studies

INTRODUCTION

Scientifically, water is an inorganic, transparent, tasteless, odourless, and nearly colourless chemical substance (Taikan, 2019). The water that is present on the earth's surface is broadly of two types' *i.e,* Freshwater and Sea water (Laurette *et al.,* 2020). The physiochemical properties and factors of the water directly affect the aquatic flora and fauna. For the aquatic ecosystem to remain balanced, it is mandatory for these factors to remain consistent to support life (Medeiros *et al.,* 2010; Bogotá-Gregory *et al.,* 2020). Sea water contains some amount of salts or sodium chloride dissolved in it whereas freshwater contains these salts in a lesser concentration. Eroding activity of the components of ocean floor, results in releasing some salts in the water thus making it salty and so-called sea water (Busch *et al.,* 2020). Increase in the salt concentration to freshwater bodies is contributed from the rains and other factors like runoffs which ultimately affects aquatic ecosystem by making it more acidic (John, *et al.,* 2019). Fishes are primitive chordates of aquatic habitat. They require consistent, optimum and confined conditions. The factors which contributes towards the growth and development of fishes includes pH, salinity, hardness, dissolved oxygen (DO) and temperature (Medeiros *et al.*, 2010; John, *et al.,* 2019). An important consideration for studying the cytotoxic effects of concentration of dissolved salts on freshwater carp Catla was the natural yield production. Alterations in the physio-chemical conditions of the water bodies have shown a remarkable decrease in the number of freshwater fishes (Juan *et al.,* 2020).

Due to oxidative stress, reactive oxygen species (ROS) are generated which leads to peroxidation of lipids, antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) are released (Ighodar and Akinloye, 2018). The tolerance value for change in salts concentration which is drawn through the experiment can be significantly employed in the branch of Pisciculture. In the present study, with the help of various assays and analytical procedures, the effect of the change in salts concentration of water was studied on freshwater carp *Catla catla*. Through the percent mortality analysis LC_{50} of the salt concentration were revealed. Other assays like lipid peroxidation and antioxidant enzyme assay and histopathological analysis was carried out to investigate the damaging effect of increasing salts concentration of the water. The damage that occurred above and during the tolerance and resistance

period was measured through lipid peroxidation and antioxidant enzyme assay. The principle objective of the study is to reveal the extent of tolerance of the carp to the change in the dissolved salts in the water. This was achieved with the help of LC50 analysis through percent mortality, biochemical method and histopathological assays.

MATERIALS AND METHODS

Maintenance and gathering of the experimental animal

During experimentation, the collection, maintenance and experimental methods recommended in the APHA were followed (APHA, Standard methods for the examination of water and wastewater 20th edition (1998). During this period, fish were maintained in 150-L capacity aquaria with water and equipped with filter and oxygenation systems. For the entire duration of the experiment, 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.

Toxicant employed

For administration as toxicant, artificial seawater (ASW) of desired concentrations: 10%, 25%, 50%, 75%, 100% was prepared. All the preparation of chemicals was done as per the analysis and assays. Initially, the order of assays and analysis to be administered was decided followed by the preparation. Stock solution of toxicant was prepared. Later, desired amount of toxicant (10%, 25%, 50%, 75%, and 100%) was added to the appropriate buckets.

Analysis of per cent mortality

Into 5 litre plastic bucket containing appropriate volume of test solution, 20 test animals were introduced in a static bioassay system. Experiments were carried out in replicates and a separate control was maintained. The fingerlings were not fed during the period of exposure. After conducting range finding tests, five different concentrations namely 10%, 25%, 50%, 75% and 100% were selected to determine the LC50 values through the analysis of percent mortality value. The formula for the mortality of a defined population, over a specified period of time, is:

Lipid peroxidation assay

Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be quantified calorimetrically following its controlled reaction with thiobarbituric acid. The measurement of these 'Thiobarbituric Acid Reactive Substances' (TBARS) is a well-established method (Devasagayam, 1986) for monitoring lipid peroxidation. Tissues of the fingerlings were obtained and homogenized in phosphate buffer followed by boiling the sample with TBA reagent for 30 minutes. Pink colours of TBARS formed were estimated at 532 nm spectrophotometrically.

Antioxidant Enzyme Assay

The activity of catalase (CAT, EC 1.11.1.6) was determined by the method described by Aebi (1984). Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to method of Beauchamp and Fridovich (1971). Glutathione peroxidase (GPx, EC 1.15.1.9) activity was measured by the method given by Lawrence and Burk (1976). Glutathione reductase (GR, EC 1.8.1.7) activity was determined according to the protocol of Goldberg and Spooner (1983). Enzymes activities were expressed as U/mg.

Measurement of protein

Protein was estimated following the method of Lowry *et al.,* (1951).

Histopathological analysis

After the time of final incubation period (96 hours) 5 fishes were selected from each bucket of experimental set including the control. Tissues of gills and liver were extracted and kept for fixation in Bouin's fixative for 12 hours (Miki *et al*; 2018). The tissues were further immersed in alcohol for clearing and removal of excess fixative. Following the Humason (1967) method of tissue processing, the tissues were processed for the xylene clearing, dehydration, impregnation and infiltration, embedding, sectioning and finally mounting. Sections of 7 µm were obtained employing the rotatory microtome (Leica RM 2235 Germany). Haematoxylin and Eosin stains were used for the preparation of the sections. The prepared sections were lastly fixed with Canada balsam.

Statistical Analysis

The mortality (%) data obtained were used to calculate the 24, 48, 72 and 96 hr LC50 values by Probit analysis method, using a statistical package (Grafpad software). ANOVA was used to compare the LC50 values of artificial sea water to test organisms after 96 hrs. All experiments were repeated at least five times and data presented is average of these replicates. One-way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance of the differences among experimental groups. All the statistical analyses were done using SPSS 17.0 software

RESULTS AND DISCUSSION

Aquatic vertebrates particularly fish; appear to have similar enzyme and receptor systems as of that in mammals (Kimberley *et al.,* 2003). By changing and adapting metabolic functions, fish react to environmental toxicants (Reddy and Rawat., 2013). Changes in the enzymatic activities of aquatic organisms as a result of their habitat fluctuations are widely used to demonstrate tissue damage and also diagnosis of fish diseases (APHA, 2005). Morphological changes, altered biochemical and histopathological framework (Bernet *et al.,* 1999), variations in the production and activity of different enzymes (Gill *et al.,* 1990) are some of the vital parameters

that show alterations and changes when the habitat is subjected or faces any imbalances (Hadi *et al.,* 2012).

Morphometric measurements and behavioral changes

The present study mainly attempts to understand and revile the effect of increasing salt concentration and dissolved ions on the biochemical and histological parameters. Morphological inspection for the quantitative measures like length, breadth, height and weight were carried out during the study (Table 1). Length of the fingerlings varies from 4.5 ± 0.6 cm; breadth from 1.01 ± 0.3 cm; height from 1.05 ± 0.3 cm and weight from 8.75 ± 0.9 gm. Increasing concentration of salts caused the fingerlings to turn bluish pink accompanied by continuous releasing of a slimy, sticky and pungent smelling fluid on the entire body surface. The distinctiveness in the behaviour was absolutely concentration dependent. Fingerlings in the water with 10% ASW exhibited a negligible behaviour while that in 25% ASW, exhibited slight modifications in the swimming pattern. No death of fingerlings was observed in the water with 10% and 25% ASW. Evidence of slimy secretions around the body surface were noticed in the fingerlings in water with 50% and 75% ASW. Fingerlings that were in the water with 100% ASW were dead within 10 minutes of introducing into the experimental setup.

Table 1: Morphometric measurements of fishes

Different Concentrations	Wt. of fingerlings (gms)	Length (cm)	Breadth (cms)	Height (cms)
Control	9.5 ± 1.2	7.2 ± 0.5	1.2 ± 0.1	1.1 ± 0.3
10 % ASW	9.2 ± 1.1	6.6 ± 0.2	2.2 ± 0.2	1.0 ± 0.4
25 % ASW	10.2 ± 1.1	7.2 ± 0.3	1.4 ± 0.4	1.3 ± 0.3
50 % ASW	10.1 ± 1.0	6.9 ± 0.8	1.3 ± 0.8	1.2 ± 0.4
75 % ASW	11.1 ± 1.2	7.8 ± 1.2	1.4 ± 0.5	1.1 ± 0.1
100 % ASW	10.1 ± 1.4	7.2 ± 0.9	1.2 ± 0.4	1.0 ± 0.2

Behavioural patterns and overall physiological responses that are exhibited by the fishes is the direct result of their habitat conditions (Butchiram *et al.,* 2013). When the fishes were subjected to different concentrations of ASW, multiple observational changes were noted including change in the color of skin, constant ejection and appearance of slimy, pungent smelling sticky fluid, restless movement and dwelling variations which indicated their resistance to the changing habitat conditions and struggle for adapting with the same.

Estimation of LC $_{50}$ through percent mortality

For the analysis of the toxic effect of a toxicant on an organism, the LC50 analysis is employed through which an approximate concentration of the toxicant upto which it exerts toxic effect on organisms can be measured (Yuniari, *et al.,* 2016; Santosh *et al.,* 2020). Percent mortality of ASW for the fingerlings of *Catla* has been summarized in Fig. 1. The mean percent mortality was recorded at 50% concentration of ASW whereas the lowest mortality was observed in 10% ASW and highest in 100% concentration as compared to that of control. The LC_{50} value that was drawn via percent mortality was found to be 47.5% which suggests that the tolerance for increase in the salt concentration is adaptable up to this value but proves lethal when exceeded above it. Fig. 1 represents the graphical explanation of the percent mortality.

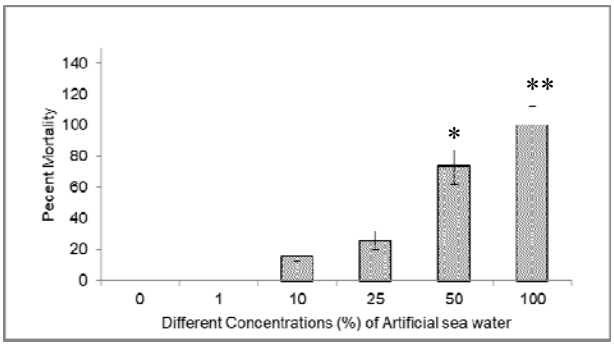

Fig.1: Percent mortality of *Labeo rohita* fingerlings exposed to different concentrations of artificial sea water
*** Statistically significant (p<0.001) compared to control group.*
** Statistically significant (p<0.05) compared to control group.*

By the per-cent mortality analysis, the concentration of ASW upto which fingerlings shows maximum tolerance was reviled. It was found that increasing the dissolved salts and ions upto 47.5% ASW was tolerated by the fingerlings and with increase in concentrations of ASW, high mortality rate was observed. The present study revealed that the mortality rate is directly proportional to the concentration of concentration of ASW.

Evaluation and measurement of lipid peroxidation in gill and liver tissue

Peroxidation of lipids is important for aquatic animals as it contain greater amounts of highly unsaturated fatty acid than other species (Antonio *et al*, 2014) has been reported to be major contributor to the loss of cell function under oxidative stress (Giulia *et al.,* 2016) and has usually been indicated by TBARS. The liver tissue lipid peroxidation was demonstrated by the increase in their respective TBARS levels as well as inhibition of the indigenous antioxidant enzyme after the artificial sea water experiment. Lipid peroxidation was maximally observed in fingerlings that were incubated in water with 100% artificial sea water with respect to control. It was observed that the rate of peroxidation of the lipids is concentration dependent. Lipid peroxidation was maximally observed at 100% ASW (liver: 7.12 nmoles ± 0.04 nmoles of TBARs/100 mg tissue $p<0.001$ and gills 8.90 ± 0.21 nmoles of TBARs /100 mg tissue; $p<0.05$) with respect to controls (liver 1.09 ± 0.04 nmoles of TBARs nmoles/100 mg tissue and gills 1.12 ± 0.1 nmoles of TBARs nmoles/100 mg tissue) respectively (Fig. 2). . Lipid peroxidation levels in the liver and gill tissue of the *Catla catla* fingerlings was significantly ($p<0.001$) increased with an increase in concentration of artificial sea water.

Analysis of the activity of antioxidant enzymes

CAT and SOD have a remarkable importance for aquatic organisms because these antioxidant enzymes protect them from free radicals that cause oxidative stress. The present study the levels of SOD and CAT activities was significantly increased with an increase in ASW concentrations. Enzymes activities are expressed as U/mg. At 100% concentration of ASW, the activity (U/mg protein) of antioxidant enzymes significantly ($p<0.001$) altered by 5.3 fold in catalase (3215± 44), 4.9 fold in SOD (2221 ± 23), 0.2 fold in GPx (0.4 ± 0.1) and 0.23 fold in GR (0.3 ± 0.1) respectively in liver tissues of fingerlings (Table 2)

compared to control. Similarly, these levels were significantly ($p<0.05$) modulated to 13.5 fold (catalase), 15.5 fold (SOD), 0.21 fold (GPx) and 0.16 fold (GR) respectively in the gill tissues (Table 3) in comparison with the control group.

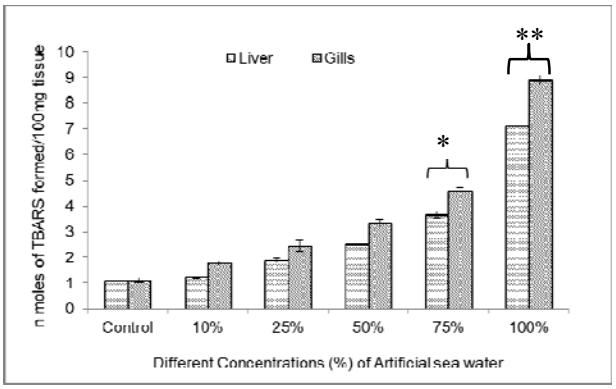

Fig. 2: Effect of artificial sea water on lipid peroxidation in liver & gill tissues

*** Statistically significant ($p<0.001$) compared to control group.*

** Statistically significant ($p<0.05$) compared to control group.*

Table 2: Specific Activity (U/mg) of antioxidant enzymes in liver tissues under different conditions

Different Conditions	CAT	SOD	GPx	GR
Control	602 ± 15	445 ± 22	2.0 ± 0.3	1.3 ± 0.3
10% ASW	826 ± 21	505 ± 23	1.5 ± 0.2	1.2 ± 0.1
25% ASW	1089 ± 22	656 ± 17	1.3 ± 0.2	1.1 ± 0.1
50% ASW	1328 ± 15*	900 ± 28*	1.2 ± 0.1*	1.0 ± 0.1*
75% ASW	1896 ± 33**	1256 ± 27**	0.8 ± 0.2**	0.5 ± 0.1**
100% ASW	3215 ± 44**	2221 ± 23**	0.4 ± 0.1**	0.3 ± 0.1**

Values are means ± SE of five individual observations. ASW: Artificial Sea Water,
**statistically significant ($p<0.001$) compared to control group;
*statistically significant ($p<0.05$) compared to control group

Table 3: Specific Activity (U/mg) of antioxidant enzymes in gill tissues under different conditions

Different Conditions	CAT	SOD	GPx	GR
Control	164± 25	104 ± 22	1.4 ± 0.3	1.2 ± 0.1
10% ASW	412 ± 33	189 ± 18	1.3 ± 0.1	1.1 ± 0.2
25% ASW	644 ± 36	289 ± 14	1.2 ± 0.2	1.0 ± 0.1
50% ASW	1118 ± 24*	612 ± 44*	0.9 ± 0.2*	0.7 ± 0.1*
75% ASW	1656 ± 21**	1025 ± 28**	0.5 ± 0.1**	0.3 ± 0.1**
100% ASW	2214 ± 20**	1618 ± 35**	0.3 ± 0.1**	0.2 ± 0.1**

Values are means ± SE of five individual observations.
ASW: Artificial Sea Water,
*** statistically significant ($p<0.001$) compared to control group;*
** statistically significant ($p<0.05$) compared to control group*

The first line of defense system (SOD-CAT) against oxidants varied with respect to the response of fish antioxidant defense system is related to cope and counteract with the toxicity of hardness and metal exposures (Atli and Canli, 2010; Venkatramalingam *et al.,* 2020). The present study is concomitant with the study of Li *et al* (2016) wherein, the levels of lipid peroxidation, CAT and SOD in fish gill were increased whereas significant inhibition of the antioxidant enzymes GPx and GR level and Na+, K+-ATPase activity was noticed in the rainbow trout *O. mykiss* after prolonged exposure propiconazole, (Li *et al.,* 2011; Abhijith *et al.,* 2016).

Glutathione reductase catalyzes the reduction of glutathione disulfide to the sulfhydryl from glutathione, which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. Decreased level of GSH level could be due to loss of adaptive mechanisms and the oxidation of GSH to GSSH (oxidized GSH) (Manjit *et al.,* 2020; Anisa *et al.,* 2020).When fish tissues are in contact with the toxicant these were removed by conjugation with GSH directly or by means of GSTs, which decreased GSH levels (Manisha *et al.,* 2019). In the present experiment significant decrease in GPx and GR activities was observed with an increase in the concentration of ASW. Glutathione reductase (GR) has been suggested as a good biomarker for oxidative stress in fish (Mukherjee *et al.,* 2017).The activity of CAT and SOD increased with the increasing concentration of ASW whereas GPx and GR showed a significant fall in their activity with an increase in the concentration of ASW.

Histopathological analysis

Histopathological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organs such as the gills, liver and kidney (Salamat *et al.,* 2016). In this study, the liver and gill histology of the fresh water fingerling *Catla catla* was analyzed.

Observations for liver tissue

Control group (Fig. 3 Fig. a) showed normal architecture and well-defined histological structures without any sign of vascular changes. Fingerlings incubated in water with 10% concentration of ASW (Fig. 3 Fig b) had vascular congestion and sinusoidal dilation compared to liver tissue to that of control.

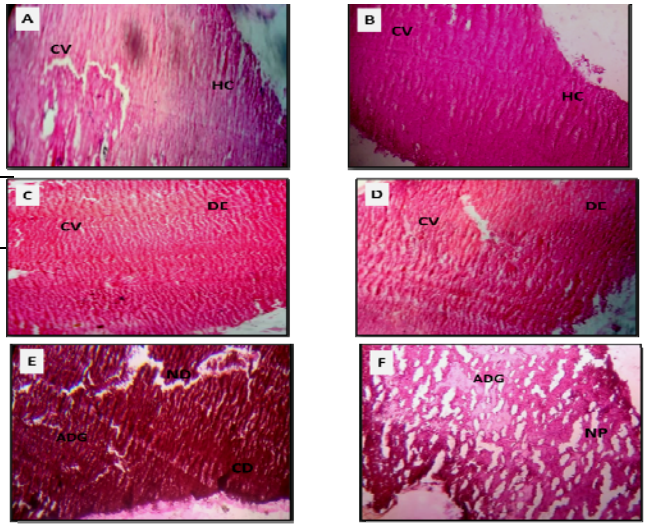

Fig.3: Histopathology of Liver tissues under different conditions (40X)

Histological changes of Liver in Catla fingerlings under different conditions Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40 X) (A) Control; (B) 10% ASW (C) 25% ASW (D) 50% ASW (E) 75% ASW (F) 100% ASW. ASW – Artificial sea water, HC- Hepatocyte, CD- Cytoplasmic degeneration, DE-Damaged epithelium, NP-Nuclear pyknosis, CV- Cytoplasmic vacuolation, ND-Nuclear degeneration, ADG-Accumulation of dark granules, CN- Cellular necrosis.

Tissues in water with 25% concentration of ASW (Fig. 3 Fig c) showed mild sinusoidal dilation and reduction in nuclear enlargement, reduced infiltration. 50% and 75% incubated (Fig. 3 Fig. d, e) exposed pronounced hepatocellular degeneration characterized by cloudy swelling and necrosis with inflammatory infiltration, dilated

central vein and sinusoidal congestion. Extensive vacuolation of hepatic cells with several foci of coagulative necrosis, blood congestion and accumulation of dark granules was observed in tissues incubated in 100% ASW (Fig. 3 Fig. f).

Observations for gill tissue

The gill tissues of the fingerlings from the control set (Fig. 4 Fig. a) exhibited normal morphology. Primary lamella projects from the posterior edge of gill arch. Secondary lamella originates on superior and inferior surface of primary lamella. Gill arches showed clear boundaries of hypertrophic zone, growth zone, and apical zone which is supported by mucosal epithelium. Normal distribution of macrophages, endothelial cells, mucous cells, and chloride cells was observed. Observations on gills of fingerlings incubated in water with concentration of ASW concentration 10% and 25% (Fig. 4 Fig. b and c) had showed the normal architecture as to that of control. Tissues from the concentration of 50% and 75% (Fig. 4 Fig. d and e) exhibited clumping of cells and cell debris, detachment of epithelial surface in primary gill lamella, gill arch disintegrated mass of spongy cartilage with distorted articles. Tissues from the concentration of 100% ASW exhibited degenerative, necrotic proliferative changes in gill filaments edema in gill filaments and secondary lamellae and congestion in blood vessels of gill filaments (Fig. 4 Fig. f).

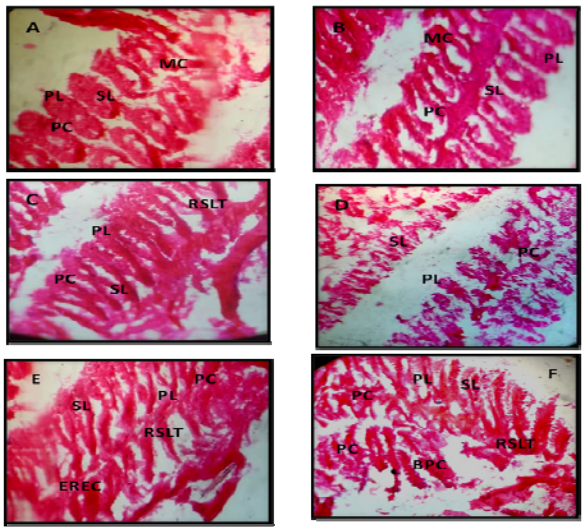

Fig. 4: Histopathology of Gill tissues under different conditions (40 X)

Histological changes of Gills in Catla fingerlings under different conditions: Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40 X). (A) Control; (B) 10% ASW (C) 25% ASW (D) 50% ASW (E) 75% ASW (F) 100% ASW. ASW Artificial Sea Water, PL- Primary lamellae, SL- Secondary lamellae, PC- Pillar cells, BPC- Breakdown of pillar cells, LSGE- Lifting of secondary gill lamella epithelium, CSL- Curling of secondary lamellae, RSLT- Rupture of secondary lamellae tip, RBPC- Rupture and breakdown of pillar cell system, EREC- Oedema and rupture of epithelial cells.

Inferences of this study showed mild to moderate hyperplasia after acute and chronic exposure of Catla fingerlings to different increasing concentrations of artificial sea water. Histological observations on gills of fingerlings infected with different concentrations of ASW revealed fusion and loss of secondary lamellar epithelium. These pathological changes may be a reaction to ions of the ASW intake or an adaptive response to prevent the entry of the pollutants thorough the gill surface (Dercia *et al.,* 2019). Similarly the liver tissue exposed to different concentration of ASW exhibited several pathological changes including hyperplasia, degeneration of blood vessels, vacuolisation, hypertrophy; pyknotic nuclei, necrosis, and accumulation of blood vessels. The observed alterations in gills and liver tissues indicates the defense mechanisms, since these results in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants (Das *et al.,* 2018).

CONCLUSION

The present study reveals that the alteration in the antioxidant enzymes activities and histopathology of the freshwater fingerlings as a result of their exposure to various increasing levels of toxicant can be considered as a tool for biomonitoring of a particular freshwater aquatic environment. Taken together the study clearly suggests the importance of selecting the sensitive biomarkers in appropriate tissues for biomonitoring toxicity in an aquatic environment. If the present rate at which they are introduced into water bodies is not monitored, existences of aquatic organisms in water bodies are in serious threat. LC50 value in the present study can further be administered by ecologists, toxicologists and fish farms as a monitoring parameter of any respective freshwater rivers.

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