Pakistan J. Zool., vol. 57(4), pp 1967-1976, 2025 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20230509070520

Histo-morphometric Indices and Catalase Response in Muscles, Gills and Intestine of Cadmium Stressed *Hypophthalmichthys molitrix*

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Article Information
Received 09 May 2023
Revised 05 February 2024
Accepted 16 February 2024
Available online 09 August 2024 (early access)
Published 05 July 2025

Authors' Contribution
RA executed the research work. HN deigned the research work. KM and NUR helped in visual analyses of slides. TA, MB and WS helped in writing article. SAQS and MU facilitated in lab work. SI and MUI helped in statistical analyses.

Key words
Antioxidant enzyme, Freshwater fish, Gills, Intestine

ABSTRACT

The experiment was done to assess at the impact of cadmium (Cd) on histology and antioxidant enzyme (catalase) activity in different tissues (gills, intestine, and muscles) of freshwater fish, *Hypophthalmichthys molitrix.* For this purpose, acute toxicity of Cd to *H. molitrix* was determined. Several concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5, 35, 37.5 and 40 mgL^{-1}) of Cd were given to fish for 96-hr. The LC_{50} and lethal concentration (96-h) of Cd was found to be 20.661 and 42.801 mgL^{-1}, respectively at 95% confidence interval. Histological examination of gill tissues showed fusion and curling of secondary lamella, degeneration of epithelium of gills and vasodilation in gill filaments. In intestinal tissues, histological alterations including sloughing and degeneration of epithelial cells of intestinal villi were observed. Moreover, significant ($p<0.05$) increase in height, width and muscularis mucosa of villi and the significant decrease in the crypt depth and tunica mucosa in intestinal tissues of treated fish were exmined. The muscles of fish showed atrophy, muscle fiber's degeneration and reduction in diameter of muscle fibers were observed. Biochemical analysis showed significant decline ($p<0.05$) in catalase activity in muscles, intestine, and gills of Cd treated fish. In conclusion, histological and enzymatic changes induced by heavy metals exposure are useful for assessing the harmful impacts of toxicants in various species of fish.

INTRODUCTION

The ecological and public health issues brought by heavy metal contamination are receiving more and more attention (Sadeghi *et al*., 2015; Pi *et al*., 2016). Due to land-based activities, a significant amount of heavy metals has been accumulated across the marine environment (Javed *et al*., 2017; Shah *et al*., 2020). A range of fish species are used as indicator to assess the health of both ecosystem and aquatic life as a result of the daily rise in metal pollution (Abbas and Javed, 2016).

Gills, skin, ingestion and adsorption are the routes from where heavy metals may get entry into a fish's body (Ahmed *et al*., 2014; Batool *et al*., 2021) and due to the absorption of harmful metals, these organs may also experience histopathological changes (Hanna *et al*., 2005).

The non-essential heavy metal cadmium is extremely hazardous to aquatic life (Van Dyk *et al*., 2007) and its persistent nature makes it possible to accumulate in the

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0030-9923/2025/0004-1967 $ 9.00/00

environment (Javed, 2005) and may cause changes in quality of water (Andhale and Zambare, 2012). Aquatic life eventually faces a risk from accumulating Cd in aquatic environments (Cao *et al*., 2010; Rana, 2014). Studies on a variety of fish species suggest that cadmium may disrupt biochemical and physiological reactions in tissues (Al-Asgah *et al*., 2015). According to studies on fish exposed to cadmium, the liver, kidney, intestine, gills, and muscle accumulate large levels of the metal (Kim *et al*., 2004; Cogun *et al*., 2003; Kalay and Canli, 2000). The organ structural damage and altered enzyme activity have both been connected to its buildup in fish (Naik *et al*., 2020).

The histopathological alterations brought by heavy metal exposure are useful in assessing their harmful effects on various fish species (Clemente *et al*., 2013). Intestinal walls are the sites for the absorption of cadmium in freshwater fish (Jayakumar and Vattapparumbil, 2006). Previous studies showed histological changes in fish intestine such as atrophy of mucosa muscularis, necrotic alterations in mucosa and sub mucosa, and deterioration of the intestine's lumen cells (Kaoud *et al*., 2013; Padrilah *et al*., 2018) after exposure of heavy metals. Degenerative alterations in the muscle tissues have also been observed as a sign of exposure to harmful substances like heavy metals (Kaur *et al*., 2018).

One of the unavoidable features of aerobic life is oxidative stress. In living organisms, antioxidant defenses and reactive oxygen species (ROS) are synthesized in an unbalanced manner, which results in oxidative stress (Nishida, 2011). For survival, an appropriate ROS balance must be maintained. The cellular antioxidant system, which includes the enzymes glutathione peroxidase (Gpx), superoxide dismutase (SOD), catalase (CAT), thioredoxin reductase (TrxR), and peroxiredoxins (Prx), controls the balance of ROS in the body. One of these key enzymes, CAT, is responsible for catalyzing the transformation of the hazardous hydrogen peroxide (H_2O_2) into the non-toxic byproducts water and oxygen (Nordberg and Arner, 2001).

The electron transport pathway in mitochondria is blocked by cadmium, which also increases the formation of ROS (Wang *et al*., 2004). Another redox inactive metal, cadmium, affects antioxidant enzymes and depletes the primary antioxidants in cells, causing the production of ROS (Ercal *et al*., 2001).

The silver carp (*Hypophthalmichthys molitrix*), is a significant fish economically, and serves as a model organism (Liu and Xie, 2003). It is more vulnerable to cadmium than other Chinese carps (Pi *et al*., 2016). Therefore, this study was conducted to assess the impacts of cadmium on silver carp to better understand the histological and biochemical changes that occur.

MATERIALS AND METHODS

Fish collection and acclimatization

This study was conducted under the laboratory conditions in the Department of Zoology at CUVAS, Bahawalpur, Pakistan. Silver carp (*Hypophthalmichthys molitrix*) 150 days old having weight 180–200 g, were collected from Fisheries Research and Training Complex, Bahawalpur. The live fish was brought to the laboratory in well aerated plastic bags, acclimatize to the lab environment for 10 days before using them in experiment.

Acute toxicity test

Each dose included three replicates overall, and there was also a control group. A total of 10 fish individuals per aquarium were distributed against cadmium metal. Different concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, 32.5, 35, 37.5 and 40 mgL^{-1}) of cadmium were given to fish to find out the acute toxicity value for 96-h. The mortality rate was counted in intervals of 12-h and the aquariums' dead fish were taken out. The 16 h before and during test fish was not fed.

Histological studies

For histological study, gills, intestines, and muscles of control and experimental fish were taken. For light microscopic examination of gills, intestine and muscles sample, paraffin-embedding technique was used reported by Bancroft *et al*. (2013).

Analysis of catalase activity

The pooled samples of gills, intestine and muscles were washed in ice-cold phosphate buffer solution blotted and weighed. By using Teflon homogenizer, 4 volumes of phosphate buffer (pH 7.4) were used to homogenize the tissues. Centrifugation of the resulting homogenate was done for 15 min at 16,000 rpm and 4°C. The supernatant was pouring out and at –20 °C, it was stored until analysis. The catalase (CAT) was assayed using spectrophotometer at 240 nm by adopting the procedure of Chance and Mehaly (1977).

Statistical analyses

To find out the 96-h LC_{50} and lethal concentration of cadmium, Probit analysis was performed for *H. molitrix.* For histological analysis, obtained data was statistically analyzed by applying t-test for multiple comparisons. For catalase activity comparison, One-way analysis of variance (ANOVA) was applied to find out the statistically significant differences among studied variables. At $p<0.05$, statistical significance was determined.

RESULTS AND DISCUSSION

Acute toxicity

The recent investigation demonstrated that when the metal content increased, fish mortality increased. For *H. molitrix*, the 96-h LC_{50} and lethal concentration of cadmium was found to be 20.661 and 42.801 mgL^{-1}, respectively. According to Pandey and Madhuri (2014), fish species and habits affect heavy metal toxicity. Several authors (Chandra and Verma, 2021; Yalsuyi *et al*., 2017; Vajargah and Hedayati, 2017; Hedayati, 2016; Pi *et al*., 2016) have reported the toxicity of Cd to various fish species. According to Abdel-Warith *et al*. (2011), there is a direct relation of fish (tilapia) mortality with increasing concentration of metal (Zn), fish mortality rose in a dose-dependent way. A more precise endpoint for assessing the impacts of metal exposure would be fish mortality (De-Schamphelaere and Janssen, 2004). Additionally, determining acute toxicity is typically the first step in assessing and evaluating the toxic properties of all compounds (Akhila *et al*., 2007).

Histological studies

Table I shows intensity of histological changes in gills, intestine and muscle tissues of fish.

Table I. Intensity of histological alterations in gill, intestine and muscle tissues of *H. molitrix* after treatment with cadmium.

Tissue	Parameters	Control	Treated
Gills	Fusion of secondary lamella	-	++
	Degeneration of epithelium and secondary lamella	-	+++
	Curling of secondary lamella	-	++
	Vasodilation	-	+++
Intestine	Epithelial cells of villi	-	++
	Degeneration of epithelial cells	-	+
Muscles	Muscular atrophy	-	++
	Degenerated muscle fibers	-	+++

Normal (-); Mild (+); Moderate (++); Severe (+++).

Gill

In cadmium treated gills of fish, histological alterations in the form of fusion and curling of secondary lamella, severe degeneration of epithelium and secondary lamella and severe dilation of blood vessels in gill filament (Fig. 1) was noted in comparison to control group. Massive pillar, mucous and epithelial cell degeneration as well as damaged gill epithelium, loss of mucous cells, an increase in the number of vacuolated cells, and damaged gill epithelium were all reported in the gills of Cd treated freshwater fish, *Channa Punctata* (Verma *et al*., 2020). Naz *et al*. (2021) noted the telangiectasia, necrosis, and lamellar epithelial cell atrophy in the gill of *Catla catla*. The main site for metal absorption are gills, however the intestine's epithelium may also be involved (Mohamed, 2008). Similar findings in the gill tissues of *Clarias Batrachus* were reported by Selvanathan *et al*. (2013) including disruption of gill filaments, higher the mucus cells with secondary lamella fusion, cell separation from pillar system and hyperplasia on epithelial cell surfaces. Moreover, changes in lipid vacuolization, separation of the respiratory epithelium and edema was also observed in secondary lamella. In the secondary lamella of Cd-treated *Oreochromis niloticus*, Mekkawy *et al*. (2013) observed sub-epithelial edema, and hypertrophy. Similarly, Ahmed *et al*. (2014) observed the necrosis, secondary lamellae fusion and hypertrophy of the mucous cells in gills of freshwater climbing perch *Anabas testudineus* after cadmium exposure.

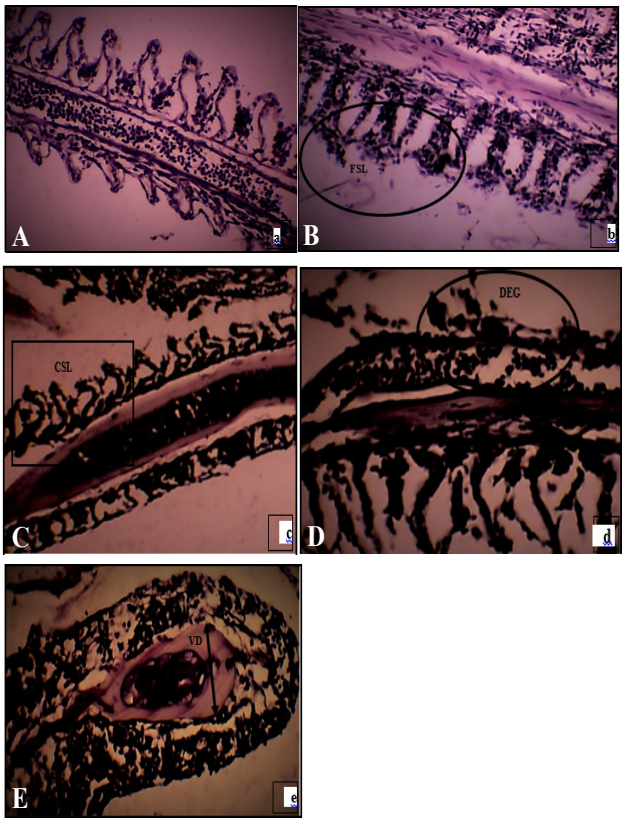

Fig. 1. Histological structure of gills of fish *H. molitrix* exposed to Cd. A, Normal gill structure. B, Fusion of secondary lamella (FSL). C, Curling of secondary lamella (CSL). D, Degeneration of epithelium and secondary lamella (DEG). E, Vasodilation in gill filament (VD). Stain: Hematoxylin and eosin; Magnification: 10X

Therefore, it can be suggested that the epithelium of gills served as the main site for the entry of pollution and, when it interacts with metals, multiplied, leading to hyperplasia. Additionally, fish gill lesions may also be observed as defensive measures taken by the fish because lesions increase the length of time it takes for dissolved metals to enter in the bloodstream (Butchiram *et al*., 2013; Akpakpan *et al*., 2014; Javed and Usmani, 2015).

Intestine

In the intestinal tissues of Cd treated fish significant increase in villus height, width and muscularis mucosa and the significant decrease in the crypt depth and tunica mucosa was observed as compared to control group (Fig. 2). Moreover, sloughing and degeneration of epithelial cells of villi was observed in Cd treated fish (Table I, Fig. 2). Uncontrolled releases of the potentially hazardous element Cd can affect fish's intestinal structure or impair their ability to absorb nutrients (Chang *et al*., 2019). The intestinal histological lesions in yellow catfish (*Pelteobagrus fulvidraco*) include enlarged goblet cells, increased mucus, vacuolization, and thicker lamina propria exposed to Cd (Xie *et al*., 2019). Naz *et al*. (2021) noted the villi atrophy, epithelial villi sloughing, and villi congestion after cadmium and copper exposure in the intestinal tissues of *C. catla*. Metals are primarily absorbed by the gills, but they can also be taken up by through the intestinal epithelium (Mohamed, 2008). Histological changes examined by Kaoud *et al*. (2011) in the *O. niloticus* intestine including edema, degenerative alterations in mucosa and submucosa, necrotic changes in the lumen of intestine and deterioration in the submucosa and muscularis mucosa after exposure to cadmium. Cadmium toxicity was also observed in *O. niloticus* by Omer *et al*. (2012) including inflammatory cells infiltration, blocking of blood vessels of submucosa and marked desquamation in the intestinal mucosa. Fish *O. niloticus* showed histological changes in intestine like degeneration in mucosa and atrophy of muscularis when cultured in heavy metals polluted water of Nile River (Kaoud and Eldahshan, 2010). Gaber *et al*. (2014) also found same results for *Dicentrarchus labrax* and *Sparus aurata* captured from heavy metal polluted Bardawil lagoon.

Due to the fact that it is the principal pathway through which dangerous toxicants enter the body, the digestive tract is essential as a surface for direct and indirect exposure to meals. The surface of intestine is very essential for the absorption of toxic substances, notably metals, and the histo-pathological lesions seen are closely related to this fact. The hazardous compounds can increase the permeability of cell membrane by interacting with phospholipids in membrane, and they can easily cross the surface of the villi, resulting in intestinal surface injury (Li *et al*., 2013). The Cu-exposed fish *Lates calcarife* showed the histological changes including damage to muscular layer and goblet cells, vacuolization, fusion in villus, irregularity in muscularis mucosal and excoriation in the mucous membrane (Maharajan *et al*., 2016). It was demonstrated that *Oreochromis niloticus* exposed to Cd had muscle degeneration, leukocyte inflammation, and apoptosis in several intestinal tissue nuclei (Younis *et al*., 2013). According to Saraiva *et al*. (2015) minor inflammation was observed in the fish intestines captured from contaminated water.

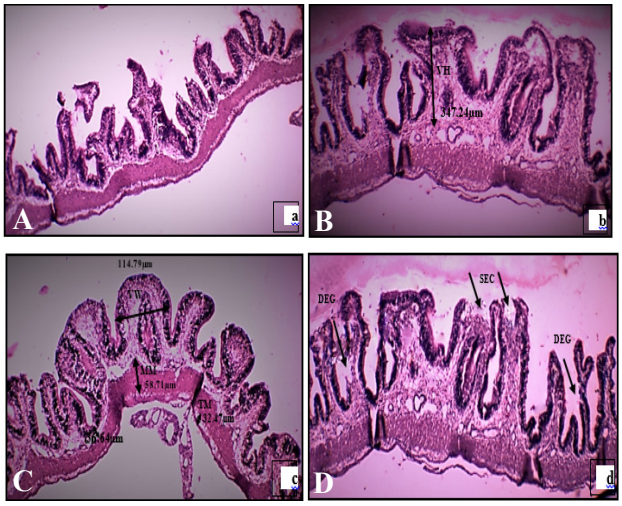

Fig. 2. Histological structure of intestine of *H. molitrix* exposed to Cd. A, Normal intestinal structure. B, Increase in villus height (VH). C, Increase in villus width (VW) and muscularis mucosa (MM) while decrease in crypt depth (CD) and tunica mucosa (TM). D, Sloughing of epithelial cells of villi (SEC) and degeneration of epithelium of villi (DEG). Stain: Hematoxylin and eosin; Magnification: 10X

Muscle

In muscular tissues of Cd-treated fish, it was noted that significant decrease in the muscle fiber diameter decreased significantly in comparison to control fish (Fig. 3). Muscular atrophy and severe muscle fiber deterioration was seen in Cd treated fish (Table I, Fig. 3). Shortening and elongation of muscle bundles of *Labeo rohita* were demonstrated by Kaur *et al*. (2018) due to mixture of metals. In Cd-treated Zebra fish, Al-Sawafi *et al*. (2017) noticed obvious tissue chaos in skeletal muscles. They observed skeletal muscle, different degrees of swelling and high necrosis. Intracellular edema, marked thickening and separation of muscle bundles was reported in cadmium and lead treated *C. carpio* (Patnaik *et al*., 2011). Mohamed (2009) reported the atrophy, necrosis and vacuolar degeneration

in muscular bundle of tilapia exposed to contaminants. In addition to this, amassing of inflammatory cells between muscle bundles was also noted. The most eatable body part of fish is muscles but unfortunately, they are direct in contact with toxicants present in water (Sitohy *et al*., 2006; El-Serafy *et al*., 2005). Fish living in contaminated water showed epithelial lesions in their muscle tissue, which meant that they were likely being invaded by microbes that might lead to muscle bundle degeneration and extensive epidermal pathology (Saad *et al*., 2012). The immune system of muscle also act as a indicator of stress because they are very sensitive to pollutants (Dyrynda *et al*., 1997; Sauves *et al*., 2002).

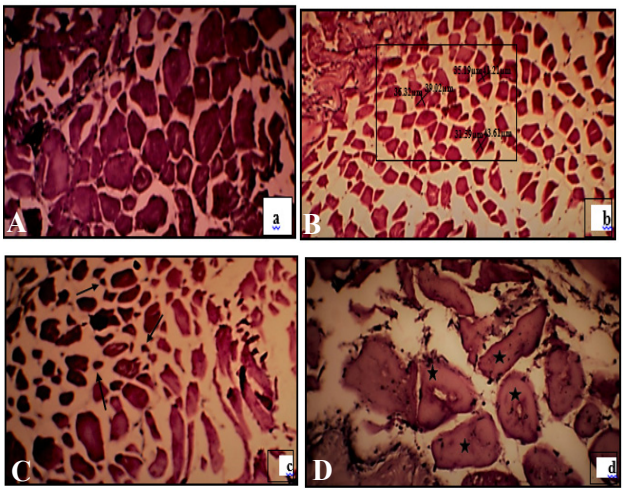

Fig. 3. Histological structure skeletal muscle of *H. molitrix* exposed to Cd. A, Normal structure of muscle fibers. B, Decreased diameter of muscle fiber. C, Atrophic muscle fibers (arrow). D, Degeneration of muscle fibers (star). Stain: Hematoxylin and eosin; Magnification: 10X

CAT activity

Results showed a significant decrease in the activity of CAT in gills, intestine and muscles of fish treated with Cd in comparison to control group, however more decline in the activity of CAT was observed after exposure of 2 days to cadmium as compared to 4 days (Fig. 4). Similarly, decreased CAT activity in the gills following exposure to various cadmium chloride concentrations in *Oreochromis niloticus* was observed by Abd-Allah *et al*. (2019). Naz *et al*. (2018) observed the significantly lower CAT activity in muscles and gills of *Cirrhinus mrigala* resulting from mixture of heavy metals. Activity of CAT in muscle tissues of *Rutilus rutilus caspicus* decreased as a result different concentration of cadmium and lead (Raeisi *et al*., 2015). Rajeshkumar *et al*. (2017) also noted reductions in muscles and gills catalytic activity of *Cyprinus carpio* on exposure

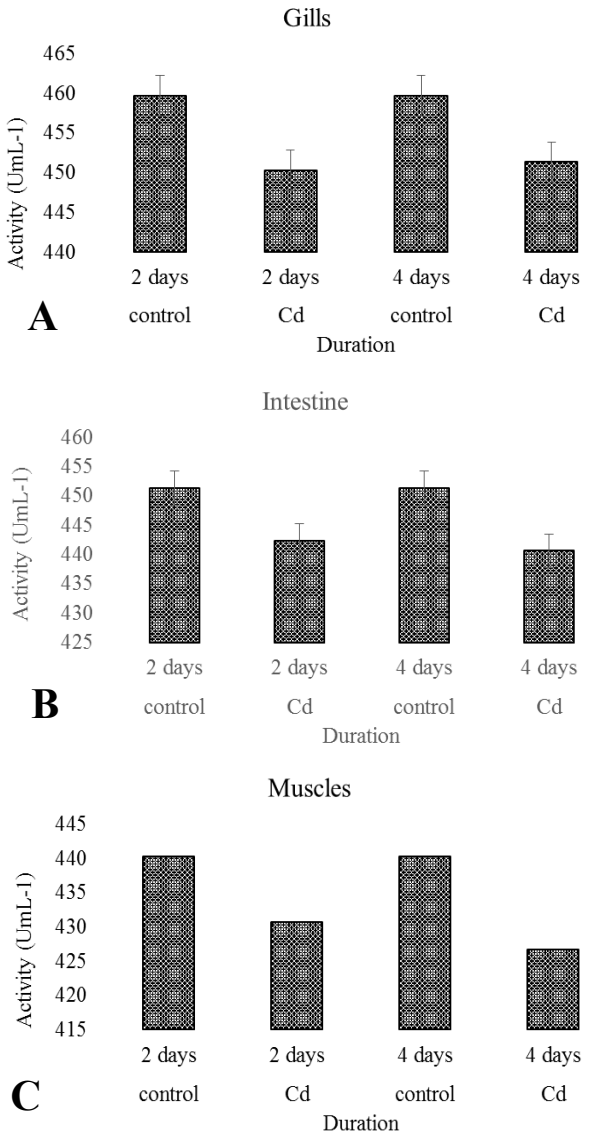

Fig. 4. Activity of catalase in gills (A), intestine (B) and muscles (C) of *H. molitrix*.

to Cr+Pb+Cd. Decline in the activity of catalase in kidney of *O. niloticus* was observed by Ahmed *et al*. (2016) after exposure to mixture of Pb+Cd. Decreased catalase activity was observed by Latif and Javed (2019) in gills, muscles, kidney, brain and liver of major carps after exposure to metals mixture (Cd+Cr+Cu+Pb) for 96-h. Mahamood *et al*. (2021) demonstrated that the exposure of different metals to *Labeo rohita* caused significant decline in bronchial and hepatic tissues catalase level. Environmental conditions, exposure time, and toxicant type can all effect how the CAT reacts (Atli and Canli, 2010). Arshad *et al*. (2018) observed the decrease catalase activity in muscle and gills

of *Channa striata* exposed to Pb+Ni mixture. Rehman *et al*. (2021) also noted the same results in gills of tilapia. The activity of intestinal antioxidant enzymes could be dramatically reduced by prolonged exposure to $CuSO_4$, which could also harm the intestinal mucosa (Li *et al*., 2023).

Heavy metals have the ability to bind to proteins, lipids and nucleic acids (DNA/RNA), once they have entered the body. Thiol (-SH) groups are frequently used in the binding process to modify cysteine residues in proteins and enzymes. The intracellular redox equilibrium can be disturbed by this particular protein inactivation. The result is an unbalanced antioxidant defence system. Amino acids with functional group SH are utilized as ligands to combine heavy metals with thiol-containing proteins (Burford *et al*., 2005).

CONCLUSION

The findings of the current investigation indicated that the acute toxic exposure of Cd leads to damage the tissues of gills, intestine and muscles of *Hypophthalmichthys molitrix* and also have a negative impact on biochemical indices of fish, proving that cadmium could potentially be hazardous. According to this study it could be a valuable bio-monitor for determining metal pollution in a variety of aquatic habitats.

DECLARATIONS

Acknowledgement

The authors are grateful to Department of Zoology, CUVAS, Bahawalpur, for providing lab facility.

Funding

No funds, grants and other support were received for this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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