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Inter-Specific Differences in the Sensitivity, Accumulation and Antioxidant Capacities of Three Cyprinids Exposed to Heavy Metals Mixture

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ABSTRACT

The potential use of biochemical techniques along with antioxidant parameters has gained considerable attention due to their use as biomarkers to assess contaminants impact on fish at earlier level of exposure. Fish from different species has diverse ecological needs and respond differently to the environmental stress conditions. Thus, the sensitivity, bioaccumulation and antioxidant capacities of three major carps *Catla catla*, *Labeo rohita* and *Cirrhina mrigala* towards metals mixture (Cd+Cr+Cu+Pb) were determined during present research work. Sensitivity of the three fish species (120-day old) towards metals mixture was measured in term of 96 h LC_{50} and lethal concentration. These acute toxicity tests were performed at constant temperature (30°C), pH (8) and total hardness (250 mgL^{-1}). All the three fish species varied significantly in their sensitivity towards metals mixture with the *C. catla* being significantly highly sensitive followed by *L. rohita* and *C. mrigala*. After the acute toxicity tests, fish were exposed to 2/3rd of their respective 96 h LC_{50} of metals mixture for 90 days, after which amassing of metals were determined by Atomic Absorption spectrophotometer and antioxidant capacity was measured in terms of catalase activity in the gills, liver, kidney, muscles and brain. Among the fish species, *C. mrigala* accumulated significantly higher concentration of all metals as compared to *L. rohita* and *C. catla*. Bioaccumulation of all metals among the organs followed the order: liver>gills>kidney>brain>muscles, however, metals followed the trend for their accumulation: Cd>Cr>Cu>Pb. Antioxidant (catalase) activity decreased significantly in all the tissues as compared to control. Catalase activity varied significantly at $p<0.05$ among *C. catla, L. rohita* and *C. mrigala.* During present research the maximum catalase activity was observed in the liver followed by gills, kidney, brain and muscles. As a conclusion, the sensitivity, bioaccumulation and antioxidant capacities of different fish species based on their ecological and physiological differences may be useful for biomonitoring studies of aquatic ecosystems.

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Authors' Contribution
MJ conceived the idea and supervised the research work and findings. FL did the research work, analyses and manuscript write-up.

Key words
Major carps, Acute toxicity, Accumulation, Oxidative stress, Catalase.

INTRODUCTION

The natural aquatic habitats in Pakistan have become contaminated with wide-ranging pollutants, including heavy metals and their mixtures, due to indiscriminate discharge of untreated effluents originating from a diversified industrial and domestic sewage. This situation is not only creating health hazards to the aquatic organisms, inhabiting these water bodies, but also affecting the human health by consuming these contaminated fish as food. Heavy metals are regarded as the most toxic due to their ability to bio-accumulate and magnify in the natural aquatic food chains (Jabeen *et al*., 2012). Present as a mixture in ambient waters, trace metals may enter the organisms via different routes and interact with each other affecting their uptake, amassing and toxicity. The type of interaction depends on the individual metals involved, exposure concentration, availability and exposure duration, selected species and examined tissues. The co-solutes may induce either synergistic or antagonistic effects as compared to additive behavior (Altenburger *et al*., 2003). It is necessary to consider possible interactions of metal that effect their uptake and accumulation in fish tissues, which are currently ignored by water quality guidelines for site specific water quality criteria (Norwood *et al*., 2003). Conservation of indigenous cyprinids of Pakistan *viz*. *Catla catla, Labeo rohita* and *Cirrhina mrigala* in their natural habitats makes it necessary to determine their sensitivity, tolerance limits and ability to bio-accumulate metals during chronic exposure of waterborne metals mixtures.

Biological techniques are implied for the appraisal of metallic ions toxicity and its impact on fish physiology, morphology and behavioral responses. Acute toxicity tests *i.e.* 96-h LC_{50} and lethal concentration allow us to measure the sensitivity of aquatic organisms to a particular toxicant by using fish mortality as criteria to final response against a toxicant (Kazlauskiene *et al*., 1999). However,

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in natural ecosystems, fish are commonly exposed to chronic concentrations of toxicants and their mixture and to measure these chronic effects, different biomarkers are used to assess changes in the physiological phases of organisms (Kousar and Javed, 2015).

In general, heavy metals generate free radicals through different ways that depend upon the metals involved and test organisms (Vander-Oost *et al*., 2003). Increased levels of heavy metals in the fish organs produce reactive oxygen species (ROS) *viz*. H_2O_2, O^{-2} and OH^{-1} that disturb normal enzymatic pathways to cause apoptotic cell death (Cao *et al*., 2010). Antioxidant enzymes viz. catalase, superoxide dismutase, glutathione peroxidase and reductase are protein in nature and important in protection against ROS (Norberg and Arner, 2001). These different antioxidant enzymes work as biomarker of oxidative stress and they cooperate with each other to protect cells against ROS (Shaukat *et al*., 2018). Antioxidant enzyme system may respond differently to each fish species and metals (Koivula and Eeva, 2010). Among these antioxidants, activity of at least one enzyme used as biomarker of oxidative damage should be measured to make inferences about oxidative stress. By keeping all the above facts in considerations, present research work focused to ascertain the acute toxicity of metals mixture (Cd+Cr+Cu+Pb) for the three fish species *viz*. *C. catla, L. rohita* and *C. mrigala* and effects of its sub-lethal concentration (2/3rd of LC_{50}) on the bioaccumulation and antioxidant activity in term of catalase enzyme assay in the different organs of selected fish species after 90-day exposure.

MATERIALS AND METHODS

This research work was done in the laboratories of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Prior to the experiment, fingerlings of *Catla catla, Labeo rohita* and *Cirrhina mrigala* were kept in cemented tanks for acclimation for two weeks. After this acclimation period, the healthy group of 120-day old fish fingerlings of comparatively similar weights and lengths were selected for these experiments. The average wet weight (g) and average total length (mm) of *C. catla* was 14.45±1.24 and 84.68±1.45, respectively, of *L. rohita* was 13.39±1.58 and 80.28±1.86, respectively, and of *C. mrigala* was 14.12±1.92 and 97.68±2.25, respectively.

Preparation of metals mixture stock solutions

Stock solutions of $CdCl_2.H_2O$, $CrCl_3.6H_2O$, $CuCl_2.5H_2O$ and $PbCl_2.6H_2O$ were prepared, separately, by using analytical grade compounds of Sigma Aldrich in de-ionized water by following standard procedure and mixed on ion equivalence basis (1:1:1:1) for metals mixture solution.

Acute toxicity assay

The acute toxicity of metals in terms of 96 h LC_{50} and lethal concentrations for each fish species *viz*. *C. catla, L. rohita* and *C. mrigala* was determined, separately, for the metals mixture. Fingerlings (n=10) of these fish species were taken, separately, for the collection of their mortality data during 96 h exposure of each metals concentration at constant water temperature, pH and total hardness of 30°C, 8 and 250 mgL^{-1}, respectively. The control fish were kept in metal free water conditions. Each glass aquarium was filled with 35 litre water and concentration of each metal was increased gradually to avoid any stress on the fish with 50% test concentration being reached in 3 h while full concentration in 6 hours. For the estimation of LC_{50} and lethal concentration for each species of fish, each metal concentration was started from zero with an increment of 0.01 and 0.1 mgL^{-1} for low and high doses, respectively. Fresh air was continuously supplied to all the aquaria water to maintain sufficient oxygen for fish respiration.

Determination of heavy metals in the fish organs

After calculating the acute toxicity of metals mixture in terms of 96-h LC_{50} and lethal concentration for three fish species, bioaccumulation and oxidative stress in the gills, liver, kidney, muscles and brain of fish after 90-day exposure to the 2/3rd concentrations of calculated 96-h LC_{50} were assessed.

After 90 days, fish from each treatment sampled and dissected to isolate organs. Samples of the selected fish organs (wet) were digested in HNO_3 and $HClO_4$ (3:1V/V) by following S.M.E.W.W. (1989) to determine metals *viz*. Cd, Cr, Cu and Pb concentrations through Atomic Absorption Spectrophotometer (AAnalyst-400 Perkin Elmer, USA). Calibration standards for each metal were made by serially diluting stock solutions with reagent grade water and checked standards were run along with samples.

Antioxidant enzyme (catalase) assay

For the determination of antioxidant capacity of three fish species, red blood cells was removed from the selected organs (gills, kidney, liver, muscles and brain) of metals mixture treated fish by rinsing these organs with phosphate buffer of pH 6.5 (0.2M) and homogenized in cold buffer (1:4W/V) using a homogenizer. After homogenization, the organs homogenate was centrifuged for 15 min at 10,000rpm at 4ºC, clear supernatant was preserved at -4ºC for enzyme assay while residue was discarded. Antioxidant enzyme, catalase (EC 1.11.1.6) activity was determined by its ability to reduce the H_2O_2 concentration at 240nm by

following the protocol of Chance and Mehaly (1977) with little modifications.

Statistical analyses

Mean values of 96-h LC_{50} and lethal concentrations were calculated for MM treatment with 95% confidence interval by using the Probit analyses method (Hamilton *et al*., 1977) with the help of MINITAB while the data on accumulation and catalase assay were analyzed by using Statistix 8.1 computer software. The data on various parameters were analyzed statistically by using Factorial design (RCBD), Analysis of Variance and Tukey/student Newman-Keul tests.

RESULTS AND DISCUSSION

Acute toxicity of metals mixture

Statistical analyses revealed that *C. catla, L. rohita* and *C. mrigala* showed significant differences towards MM (Cd+Cr+Cu+Pb) sensitivity. The mean values of 96-h LC_{50} and lethal concentrations of MM calculated for three fish species along with their 95% confidence limits are presented in Table I. Among the three Cyprinids, *C. catla* showed significantly higher sensitivity towards metals mixture with least value (25.42mgL^{-1}) of 96-h LC_{50} followed by that of *L. rohita* (30.31 mgL^{-1}) and *C. mrigala* (37.06 mgL^{-1}). However, *C. mrigala* exhibited significantly higher average lethal concentration with the mean value of 58.33 mgL^{-1} among the three fish species for MM (Fig. 1). Kousar and Javed (2014) investigated the toxicity of As, Ni and Zn for four fish species and concluded that *Catla catla* showed highest sensitivity, followed by *Ctenopharyngodon idella*, *Cirrhina mrigala* and *Labeo rohita*. The 96-h LC_{50} values calculation is a very important factor to check the susceptibility of fish to various pollutants. The sensitivity of fish varies from species to species and metals to metals. The metal that is toxic at low level concentration to one individual may be non-toxic or less toxic to other animal at similar or higher concentration (Shah and Altindu, 2005). *C. catla* was reported to be the significantly ($p<0.05$) most sensitive species among major carps by Azmat *et al*. (2012) and also by Javid *et al*. (2007). *C. mrigala* appeared least sensitive species, however, Abdullah *et al*. (2007) showed that *L. rohita* is least sensitive among major carps under the exposure of waterborne metals. Similarly, Naz and Javed (2012) investigated the toxicity of Fe+Zn+Pb+Mn mixture in three major carps and reported that *L. rohita* was significantly least senstitive followed by *C. catla* and *C. mrigala*.

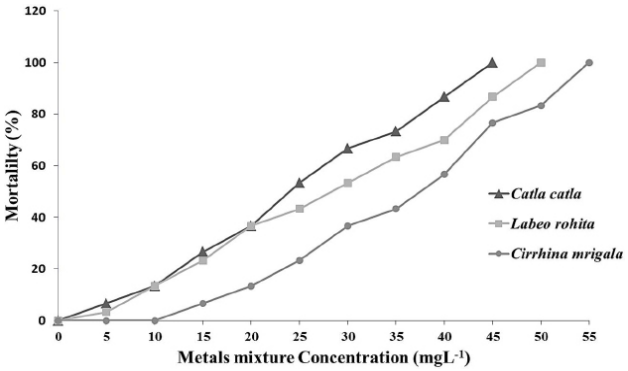

Fig. 1. Effect of different concentrations of heavy metal mixture on the mortality of three Cyprinids after 96 h exposure.

Bio-accumulation of metals in fish body

The concentration of Cd, Cr, Cu and Pb in 120-day old *C. catla, L. rohita* and *C. mrigala* body tissues were determined after exposure to 2/3rd of LC_{50} of MM and their means were presented in Table II. The amassing of all metals in the three selected Cyprinids increased significantly as compared to control. All metals showed significantly higher bio-accumulation in liver followed by gills while least concentration was found in muscles. Significant differences among the fish organs to accumulate metals are attributed to the variable physiological role played by each organ (Javed, 2012). Control fish had significantly lower concentration of these metals as compared to treated fish (Table III). *C. catla* exhibited lower (37.38±7.51µgg^{-1}) while that of *C. mrigala* showed higher (43.87±9.88µgg^{-1}) tendency to accumulate Cd in their body tissues. Chronic exposure to MM caused the liver of all fish species to accumulate significantly higher Cu (56.07±6.02µgg^{-1}), Cr (54.71±3.79µgg^{-1}), Cd (53.78±3.51µgg^{-1}) and Pb (49.99±5.75µgg^{-1}) followed by that of gills and kidney with significant differences.

Table I.- Mean 96-h LC_{50} and lethal concentrations of heavy metals mixture for three Cyprinids.

Species	LC_{50} (95% CI)	Lethal concentration (95% CI)	Regression equation (Y=a+bx)
Catla catla	25.42 (15.47-25.95)	47.19 (41.36-58.66)	Y = -2.57769+0.0869887 + 1.78240 (0.0164443)
Labeo rohita	30.31 (25.44-34.20)	53.05 (47.00-64.46)	Y = -2.88976+0.0832616 + 3.92218 (0.0148548)
Cirrhina mrigala	37.06 (32.20-40.85)	58.33 (52.50-69.73)	Y = -3.66617+0.0890386 + 2.03302 (0.0166983)

CI, confidence interval; Y, dependent variable; x, independent value.

Table II.- Bioaccumulation of metals (µgg^{-1}±SD) in different fish organs at sub-lethal (2/3rd of LC_{50}) exposure to heavy metals mixture.

Metals	Species	Gills	Liver	Kidney	Muscles	Brain
Cadmium	*C. catla*	45.69±21.97	50.86±23.34	36.77±18.79	25.12±13.94	34.45±18.30
	L. rohita	48.34±23.10	55.82±25.24	39.80±19.20	29.21±15.14	35.14±17.51
	C. mrigala	51.16±24.54	57.65±25.31	44.06±20.25	32.54±17.67	38.97±18.29
Chromium	*C. catla*	46.91±23.05	51.93±25.71	39.33±19.51	25.86±13.18	34.87±18.51
	L. rohita	51.20±24.88	55.69±24.98	45.47±22.98	34.69±19.94	44.83±22.27
	C. mrigala	54.33±23.50	59.51±25.47	52.73±24.86	36.60±18.15	49.23±24.49
Copper	*C. catla*	43.41±22.38	50.36±24.32	35.24±17.75	21.49±12.32	29.71±15.23
	L. rohita	49.52±24.45	58.85±27.18	42.09±21.24	26.45±14.33	34.52±16.59
	C. mrigala	54.57±24.21	62.01±27.44	46.52±22.99	29.96±13.74	39.07±17.49
Lead	*C. catla*	39.60±22.79	44.63±24.64	29.70±16.35	19.73±11.48	26.19±14.74
	L. rohita	46.09±24.34	52.54±25.46	39.45±20.84	23.58±13.20	33.75±18.99
	C. mrigala	50.25±24.57	56.81±26.19	43.95±21.96	26.22±14.04	38.97±19.64

Table III.- Bioaccumulation of heavy metals (µgg^{-1}±SD) in the organs of control Cyprinids.

Metals	Species	Gills	Liver	Kidney	Muscles	Brain
Cadmium	*C. catla*	6.51±0.26	5.37±0.46	3.00±0.23	0.75±0.21	2.99±0.24
	L. rohita	4.95±0.25	6.30±0.42	3.15±0.26	0.81±0.13	3.22±0.23
	C. mrigala	7.55±0.35	5.88±0.15	3.20±0.27	1.29±0.12	3.53±0.24
Chromium	*C. catla*	5.47±0.31	6.81±0.39	3.83±0.17	5.27±2.53	3.10±0.19
	L. rohita	6.70±0.48	7.53±0.24	3.14±0.19	2.12±0.15	2.83±0.08
	C. mrigala	7.36±0.11	6.54±0.29	3.45±0.16	2.49±0.22	3.63±0.27
Copper	*C. catla*	3.67±0.41	4.40±0.42	1.79±0.23	0.46±0.13	2.77±0.28
	L. rohita	7.32±0.43	5.47±0.41	1.98±0.19	0.80±0.14	3.65±0.23
	C. mrigala	5.62±0.40	5.89±0.34	2.06±0.28	2.21±0.20	3.98±0.54
Lead	*C. catla*	3.66±0.27	3.81±0.19	1.63±0.29	0.41±0.17	2.79±0.29
	L. rohita	4.82±0.17	4.97±0.19	1.63±0.18	0.80±0.15	3.24±0.39
	C. mrigala	5.11±0.23	5.42±0.37	3.70±0.27	0.98±0.15	3.85±0.34

Liver amass higher metals concentrations due to its role in bio-transformation, detoxification and higher amount of metallothionein in it while that of gills had higher tendency to accumulate metal due to their intimate contact with the contaminated water as well as effectors of ionic and osmotic regulations. Muscles in fish had significantly least concentration of Cd, Cr, Cu and Pb as 27.95±3.72, 31.38±5.73, 24.97±4.26 and 22.18±3.26µgg^{-1}, respectively. Yilmaz *et al*. (2007) reported that liver and gills of *Leuciscus cephalus* and *Lepornis gibbosus* exhibited higher tendency to amass Co, Cd and Cu while least tendency was showed by muscles due to the scarce presence of metallothionines. These results are also in agreement with the findings of Canli and Atli (2003), Yousafzai *et al*. (2012) and Squadrone *et al*. (2013).

Among all three fish species, *C. mrigala* significantly exhibited higher tendency to accumulate all selected metals in MM followed by that of *L. rohita* while *C. catla* showed significantly lower tendency to amass all metals in its body tissues. The bio-accumulation of Pb in major carps followed the order: *L. rohita* > *C. mrigala* > *C. catla* (Javidi *et al*., 2007). Exposure of three Cyprinids to Cu+Cd+Zn+Ni+Co mixture caused higher accumulation of all metals in fish liver, gills and kidney (Javed and Abdullah, 2003). Overall bio-accumulation of metals in all the three fish species followed the order: Cr > Cd ≥ Cu > Pb. All the fish species in control media showed lower amount of all metals than that of MM treated fish. Qadir and Malik (2011) observed the amassing of Cd, Cu, Pb and Cr in the tissue of eight edible fish species collected form river Chenab, Pakistan. Accumulation pattern of metals in their results was: Cr>Pb>Cu>Cd while organs in fish species followed the order as: liver>gills>kidney>muscles. These results are in line with our findings.

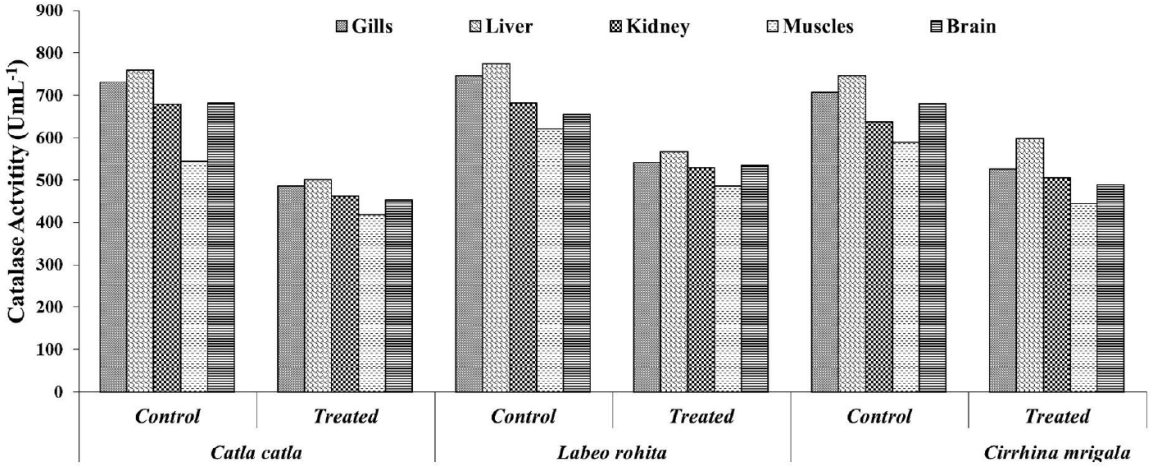

Fig. 2. Effect of heavy metal mixture on the catalase (A) and superoxide dismutase activity (UmL^{-1}±SD) (B) in different organs of three Cyprinids fishes.

Antioxidant (catalase) activity

Catalase (EC 1.11.1.6) is an important antioxidant enzyme that has main function in protecting the cells from the accumulation of hydrogen peroxide by converting it to water and molecular oxygen. The activity of catalase in the gills, liver, kidney muscles and brain of three fish species exposed to 2/3rd of their respective LC_{50} of MM is presented in Figure 2. The catalase activity inhibited significantly in all MM treated fish tissues as compared to control. Inactivation of catalase activity in all three fish species may be resulted due to the MM exposure stress as metals increase the production of reactive oxygen species. Wong and Whitaker (2002) explained that inactivation of catalase at higher H_2O_2 concentration was due to the conversion of active enzymes to inactive complexes. Effects of 20 and 80% of LC_{50} of cadmium exposure to zebrafish on catalase and glutathione activities were investigated by Sunaina (2015). A significantly decreased activities of both the enzymes were reported by them. Paul and Sengupta (2013) concluded that the lower activity of catalase in Pb treated *Channa punctatus* could be attributed to the increased production of superoxide anions that inhibit catalase activity. These results are also supported by the findings of Jamakala and Rani (2012) whose results showed decrement catalase activity in metal stressed fish.

Among the fish species, significantly higher catalase activity was observed in *C. mrigala* at MM exposure followed by *L. rohita* and *C. catla*. These species-specific variations in enzymes activity are not only depend on the taxonomic position but also on the ecological niches of fish (Rudneva, 1997). Atli *et al*. (2016) explained that antioxidant responses depend upon type of toxicants, concentration, exposure period and tissues as well as physiological and environmental factors. Trenzado *et al*. (2006) interpreted the differences in antioxidant responses between Sturgeon and Trout fish and found significantly lower antioxidant's activity in the Sturgeon due to its lower oxygen consumption in comparison to Trout. Similar studies were also done on the activities of catalase and superoxide dismutase in the carp and trout in which trout exhibited higher enzyme activities as compared to carps (Saglam *et al*., 2014).

During the present study, maximum catalase activity was observed in the liver, followed by gills, kidney, brain and muscles which agree with other literatures by Gul *et al*. (2004) and Radhakrishnan (2008). This organs-wise trend in catalase activity is also in accordance with the Hidalgo *et al*. (2002) in the rainbow trout as: liver > kidney > heart > brain > muscle. MM exposure decreased catalase activity in liver which may be due to the fact that metals bind with -SH group of enzymes and inhibit their activity. Among the organs, significantly higher enzyme activity was observed in liver because of its role in oxidative reactions and free reactive radical generations (Avci *et al*., 2005). Brain of the fish is very susceptible to damage by ROS because of its possession of highly unsaturated lipids and higher oxygen consumption. As catalase enzyme was highly activated in the liver, gills and kidney; brain and muscles were not particularly rich in antioxidants (Sahin and Gumuslu, 2004). Rajeskar and Venkatakrishnaiah (2016) analyzed oxidative stress responses in copper exposed *C. catla* and observed reduced catalase activity in the gills, liver and brain. Maximum catalase activity in the liver and minimum in brain was determined in seven animal species by Jena *et al*. (1998). Significantly decreased activity of catalase in the muscles as compared

to brain, gills and liver in vaccinated rainbow trout was reported by Tkachenko *et al*. (2014).

CONCLUSION

Inter-specific differences among *C. catla*, *L. rohita* and *C. mrigala* existed for the sensitivity towards metals mixture (Cd+Cr+Cu+Pb) in term of 96-h LC_{50} and lethal concentration. *C. catla* was found to be significantly more sensitive followed by *L. rohita* and *C. mrigala*. Sub-lethal exposure of LC_{50} caused significant amassing of all the metals in mixture in fish organs. The response of catalase activity in fish tissues of three fish species exposed to 2/3rd of LC_{50} was found to be significantly variable depending on tissues and fish species.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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