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Assessment of Heavy Metals and Antioxidant Enzyme in Different Organs of Fish from Farm, Hatchery and Indus River of Pakistan

Zeenat Bano[1,*], Sajid Abdullah[1], Waqas Ahmad[2], Muhammad Anjum Zia[3] and Wardah Hassan[1]

[1]*Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad-38040, Pakistan*
[2]*Section of Epidemiology and Public Health, College of Veterinary and Animal Sciences, Jhang, Pakistan*
[3]*Enzyme Biotechnology Lab, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan*

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Authors' Contribution
MAZ designed the study plan and statistically analyzed the data. ZB and SA collected field samples, conducted enzyme assays and estimation of heavy metals. WH collected laboratory research material and performed water analyses. WA collected research papers, composed and revised the manuscript.

Key words
Antioxidant Enzymes, *Cirrhina mrigala*, Heavy Metals, Superoxide dismutase, Catalase, Peroxidase

ABSTRACT

The aim of the present study was to determine the level of selected toxicants in water, their bioaccumulation and effects on antioxidant enzymes *i.e.* superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in vital organs (gills, liver and kidney) of fish, *Cirrhina mrigala.* The 3 different representative water samples and 60 fish samples (20 fish from each site) were collected from three sites *i.e.* fish-farm, hatchery and Indus River of Punjab, Pakistan. The results showed differences in physico-chemical parameters of water samples collected from all the sites. The concentrations of Zinc (Zn), copper (Cu) and nickel (Ni) were higher in river water as compared to farm and hatchery. Different organs of fish collected from different sites showed variations in their metal concentration. In Indus River fish, the concentrations of metals were highest in liver followed by kidney and gills. The Indus River fish also exhibited highest SOD activity in all the organs as compared to farm and hatchery fish. The activity of CAT enzyme was higher in kidneys and gills of farm fish compared to the liver of hatchery fish. The maximum POD activity was recorded in liver, kidney and gills of hatchery fish. This study reveals that fish could acquire higher uptake of metals due to excessive pollution of heavy metals and other toxic elements in river water. In response to metals toxicity, the antioxidant system can be useful as an early warning tool in natural monitoring studies.

INTRODUCTION

The natural water bodies are being polluted due to industrial, agricultural and domestic wastes leaving behind severe ecological and environmental hazards (Banerjee *et al*., 2016; Bhattacharya, 2016). The pollutants in water ecosystem produce higher contents of metal which are threatening humans and animals' health (Sekabira *et al*., 2010). Aquatic ecosystem is the natural habitat of fish and water pollution directly affects the underwater fish and human beings are indirectly at high risk by consuming these fish (Mendil *et al*., 2010; Monferran *et al*., 2016). Effluents can accumulate in living tissues for longer duration, but fish is being negatively influenced than other species (Parlak *et al*., 1999; Tapia *et al*., 2012). Zn, Cu and Ni are essential ions for the maintenance requirements of fauna and flora (Pyle *et al*., 2012). However, the excessive accumulation is definitely toxic for biota and negatively affects the aquatic life (Bielmyer *et al*., 2013; Alsop *et al*., 2014). Some heavy metals act as a cofactor or as chelates for vital metabolic reactions. Poisonous effects in the underwater fish are caused by heavy metals even at minute levels (Cohen *et al*., 2001).

Reactive oxygen species (ROS) can be produced in greater amount by aquatic organisms due to accumulation of heavy metals. The ROS such as hydrogen peroxide, superoxide radical and hydroxyl radicals cause oxidative stress in living organisms (Kurutas *et al*., 2009; Sheriff *et al*., 2014). There would be development of the defensive mechanism for ROS in living bodies. Antioxidant enzymes such as SOD, CAT and POD function in a synchronized way to counteract oxidative stress (Palermo *et al*., 2015). Antioxidant enzymes in liver, gills and kidney provide defensive mechanism against ROS, but liver is the

* Corresponding author: zeenatbano98@gmail.com
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main metabolic center that takes part in detoxification (Bagnyukova *et al*., 2006). The contaminants from water enters in fish through food, non-food particles, skin, gills and water then absorbed in blood and passed to liver for transformation or storage (Nussey *et al*., 2000; Tapia *et al*., 2012; Hashmi *et al*., 2013). Transformed pollutants are removed to excretory organs like kidney or gills (Nussey *et al*., 2000). Accumulation of heavy metals severely affects fish by disturbing their physiological, biochemical, growth and reproduction (Yilmazi *et al*., 2006). The aim of this study was to evaluate water pollution level and bio-accumulation of heavy metals in fish organs.

MATERIALS AND METHODS

Study area and sampling

The fish (*Cirrhina mrigala)* and water samples were obtained from fish-farm of Malanwan, Jhang, Government fish seed hatchery of district Faisalabad and Taunsa Barrage located on the Indus river of Pakistan (Zaidi *et al*., 2004). This fish is not listed among endangered species in International Union for the Conservation of Nature and Natural Resources (IUCN). The procedures pertaining to fish handling and processing were in accordance with the guidelines of local ethical committee of zoology and fisheries department.

Water analysis

The sample water was filtered through 0.45 µm Millipore membrane filters (Type HV), and subsequently stored in polypropylene bottles. In order to avoid metal adsorption on the inner walls of bottles, 55% nitric acid was dissolved in the water and kept at refrigeration temperature. Electronic meter (HANNA HI-9146) was used to measure temperature and dissolved oxygen contents. Electrical conductivity and pH were measured by H1-9146 and HI-8520, respectively. Further, physico-chemical analysis was carried out using standard methods as described earlier (Andaleeb *et al*., 2008). The concentrations of Zn, Ni and Cu were determined using the corresponding method numbers of each metal through Atomic Absorption Spectrophotometer (Perkin Elmer, Aanalyst-400) (SMEWW, 1989; Azmat *et al*., 2012).

Estimation of heavy metals in fish organs

The selected organs were digested with the concentrated HNO_3, and filtered through 0.45 µm Millipore membrane filter. Analysis of filtrate was carried to calculate the concentration of Zn, Ni and Cu on Atomic Absorption Spectrophotometer (SMEWW, 1989).

Profiles of enzymes

Visceral organs (liver, kidney and gills) were surgically removed from fish, washed with distilled water and rinsed with phosphate buffer saline to remove blood clots. The organs were separately homogenized with 0.2 M solution of phosphate buffer saline and centrifuged at 10,000 rpm for 15 min in temperature controlled centrifugation machine.

The biological activity of SOD was determined as described earlier (Giannopolitis and Ries, 1997). The enzyme activity was based on its ability to inhibit 50% photo-reduction of nitroblue tetrazolium (NBT). Similarly, the biological activity of CAT was determined by using the reagents and chemicals prescribed in earlier study (Claiborne, 1985), and it was assessed by measuring the reduction of H_2O_2 concentration at 240 nm of wavelength. The biological activity of POD was also measured following the previous procedure (Zia *et al*., 2011) The ability of POD to reduce the concentration of H_2O_2 judged the effectiveness of peroxidase enzyme at a wavelength of 470 nm.

Statistical analyses

Data obtained from triplicate experiments were analyzed by using the method of Steel *et al*. (1996). Analysis of variance was calculated through complete randomized design and mean values were compared by Duncan's Multiple Range test to observe the statistical differences among variables.

RESULTS AND DISCUSSION

Water analysis

Maximum values of all the physico-chemical parameters were determined in water samples collected from Indus River. Highest values of Zn, Cu and Ni were also noted in water of Indus River, whereas the minimum values of these metals were obtained in the water of fish-farm. Among metals, the concentration of Zn was highest in water samples obtained from farm, hatchery and Indus River followed by Ni and Cu (Table I).

In this study, fluctuations in pH values were due to the changes in rate of decomposition and photosynthesis, but even then these values were in accordance with the previous findings of Naik *et al*. (2015). The pH value that ranged from 6-9 is considered suitable for aquatic fauna (WWF-Pakistan, 2007). Dissolved oxygen (DO) indicates the contents of oxygen in the water for the aquatic life, and it is inversely proportional to the environmental temperature. The lower values of DO are detrimental for aquatic life and may cause death in different water bodies of Pakistan (FEPA, 2003).

Table I.- Different parameters of water analysis from three water bodies (Mean±SD).

		Sampling sites		
		Fish farm	**Hatchery**	**Indus River**
Physico-chemical parameters	Temperature (°C)	29.86±0.49b	29.93±0.55b	31.833±0.83a
	pH	7.9±0.152b	7.73±0.21b	8.03±0.12a
	Dissolved oxygen (mg L^{-1})	7.66±0.12a	6.7±0.17b	5.63±0.152c
	Electrical conductivity (mS cm^{-1})	2.16±0.02c	2.59±0.06b	2.66±0.10a
	Carbonates (mg L^{-1})	26±0.81c	67.66±0.58b	68.33±2.082a
	Bicarbonates (mg L^{-1})	119.4±0.62c	191.33±1.52b	300.86±4.25a
	Total alkalinity (mg L^{-1})	145.23±0.42c	258.9±4.04b	369.2±6.298a
	Total hardness (mg L^{-1})	112±5.00c	196.33±7.64b	238.33±7.64a
Metals	Zn (mg L^{-1})	0.05±0.02b	0.06±0.04b	2.11±0.05a
	Cu (mg L^{-1})	0.007±0.01b	0.008±0.02b	1.02±0.02a
	Ni (mg L^{-1})	0.01±0.005c	0.05±0.07b	1.23±0.22a

Means with similar letters in a row are statistically non-significant ($p>0.05$).

Deviation in the values of alkalinity confirmed that total alkalinity increased by the accumulation of bicarbonate ions. The higher concentrations of carbonates and bicarbonates in the Indus River are the ultimate cause of higher alkalinity. Total hardness is the measure of dissolved magnesium and calcium ions which are commonly responsible for the hardness of water (Naik *et al*., 2015).The data showed that the water quality of Indus River was under the influence of salt range that also brought various solids, metals, bicarbonates and salts from the hills. Electrical conductivity reflects total nutrient level in a water body that is required for an electric impulse to pass through. The higher values of conductivity shows pollution status of water body (Kim *et al*., 2001). The higher electrical conductivity in wild environment shows contamination of river water as compared with cultured environment.

Higher concentrations of Zn, Cu and Ni were noted in Indus River. The values of these metals were higher as compared to threshold levels found in earlier studies. The levels of Zn, Ni and Cu were higher than 0.086, 0.05 and 0.007 mgL^{-1}, respectively (Jabeen and Chaudhry, 2010). The level of metals in water of fish-farm and hatchery were lower than permissible range (WWF-P, 2007). In this study, the physico-chemical parameters showed better comparison with suggested standards of previous studies (USEPA, 2011) which highlighted the fitness of the fish-farm and hatchery.

Estimation of heavy metals in fish organs

Table II shows the concentrations of metals in three different organs of *Cirrhina mrigala* from three selected sampling sites. The highest concentration of metal was found in fish of Indus River followed by hatchery and farmed fish. In Indus River fish, the ionic concentrations of metals were highest in liver followed by kidney and gills. The Zn and Cu concentration in farmed and hatchery fish followed the order: kidney>liver>gills. However, the concentration of Ni was highest in liver of hatchery and gills of farmed fish.

Table II.- Metals concentration (µg g^{-1}) in different organs of *Cirrhina mrigala* (Mean±SD).

Metals	**Fish organ**	**Farm fish (n1=20)**	**Hatchery fish (n2=20)**	**River fish (n3=20)**
Zn	Liver	31.7±3.34b	33.9±3.23b	287.77±3.58a
	Kidney	42.3±3.41b	43.66±7.46b	275.71±2.39a
	Gills	26.01±6.2b	23.94±5.52b	145.85±10.23a
Cu	Liver	1.16±0.23b	1.44±0.45b	23.47±2.19a
	Kidney	2.09±0.21b	2.61±0.204b	17.35±1.41a
	Gills	0.53±0.13b	0.60±0.08b	3.04±0.72a
Ni	Liver	0.48±0.02c	1.403±0.23b	6.38±.0.18a
	Kidney	0.43±0.02c	1.25±0.13b	4.23±0.04a
	Gills	0.86±0.04b	0.81±0.1b	3.3±0.329a

Means with similar letters in a row are statistically non-significant ($p>0.05$).

Bioaccumulation of metals, like Zn and Cu was highest in metabolic organs that altered biochemical parameters for the detoxification of metals in body (Sia *et al*., 2013). Earlier findings verified the results that also showed higher accumulation of Zn in fish tissues followed by Cu and Ni (Bat and Arici, 2016). Minimum concentration of Zn and Cu was recorded in gills due to fast elimination of Zn from these tissues (Murugan *et al*., 2008). Highest value of Cu was found in kidney of cultured fish followed by liver and gills due to the fact that kidney had Cu binding cysteine rich protein. The higher accumulation in liver lowers the metal toxicity after binding with Cu binding metallothionein that also detoxifies (Malik *et al*., 2010).

Table III.- Enzymes activity (U mL^{-1}) in different organs of *Cirrhina mrigala* (Mean±SD).

Enzymes	Fish organ	Farm fish (n1=20)	Hatchery fish (n2=20)	River fish (n3=20)
Superoxide dismutase	Liver	65.60±1.14b	62.01±1.97c	73.49±0.88a
	Kidney	55.72±0.55b	57.785±1.20b	71.61±2.59a
	Gills	66.77±0.552b	65.83±9.59b	77.06±4.84a
Catalase	Liver	97.28±0.14b	102.51±0.56a	81.24±2.91c
	Kidney	82.07±0.19a	72.65±3.74b	67.84±2.05c
	Gills	92.64±0.15a	73.19±2.95b	69.01±1.22c
Peroxidase	Liver	0.466±0.011b	0.55±0.09a	0.54±0.14a
	Kidney	0.496±0.003b	0.57±0.06a	0.442±0.002c
	Gills	0.443±0.005b	0.479±0.02a	0.425±0.033b

Means with similar letters in a row are statistically non-significant (p>0.05).

Indus River's *Oreochromis mossambicus* showed Cu concentration within recommended range, while the concentration of Zn ranged from 27.4-218.8 µg/g in different tissues. The highest standard values for the concentration of Cu and Zn were 30 µg/g and 50 µg/g respectively (USEPA, 2011). Few studies have been conducted to assess bioaccumulation of metals beyond the threshold limits (Javed and Usmani, 2013). Rajeshkumar *et al.* (2013) found higher concentration of metals in fish liver at polluted site. Limited literatures are available on Ni accumulation in fish as compared to concentration and distribution pattern of other metals (Palermo *et al.*, 2015). The present research work showed safe limit for the accumulation of metals in farmed and hatchery fish for consumers while accumulation of Zn in wild fish was higher than suggested standard values that may pose Zn-induced health hazards.

Profiles of enzymes

The maximum SOD activity was noted in gills of wild fish while it was minimum in kidney of farm fish. Among organs, gills showed highest SOD activity followed by liver and kidney of fish sampled from three water bodies. The highest and lowest CAT activity was observed in liver and kidney of hatchery and river fish, respectively. The CAT activity in organs of fish from different sites followed the order: liver>gills>kidney. Maximum POD activity was noted in kidney of hatchery fish while it was minimum in gills of wild fish. Enzyme activity in organs of farmed and hatchery fish followed the order: kidney>liver>gills while in wild fish it followed as liver>kidney>gills (Table III).

The SOD catalyzes the breakdown of the superoxide anion (O_2^-) to water and hydrogen peroxide (H_2O_2) that were further detoxified by the CAT enzyme. The SOD-CAT system gives the initial defense in combating the oxygen toxicity (Sheriff *et al.*, 2014). In wild fish, the activity of SOD found highly significant due to the metal accumulation. Continuous exposure to pollution indicated the sign of compensatory tissue reaction that ultimately increased SOD activity in the fish gills and least cellular injury to fish gills. On the other hand, decreased SOD activity in hepatic tissues was observed in previous studies conducted on fish (Parthiban and Muniyan, 2011; Zheng *et al.*, 2014). A similar kind of study was conducted from various sites in the Ceyhan River, Turkey that focused on the gills and liver for antioxidant enzymes (Sahan *et al.*, 2010).

Catalase is a vital enzyme to secure the cells from lethal effects of H_2O_2 and ROS (Coban *et al.*, 2007). Reduction in CAT activity due to decreased ability to protect cells against H_2O_2 was also reported by Papagiannis *et al.* (2004). Reduction in CAT activity due to the superoxide radicals flux was reported from contaminated environment. The accumulation of metals significantly decreased the CAT activity in wild fish. Kubrak *et al.* (2013) and Karadag and Firat (2014) reported lower level of CAT activity in fish from polluted sites due to heavy metals and denaturation of H_2O_2.

The present research work showed the direct relationship between the POD activity and the levels of metal deposition. It is apparent from the results that POD activity decreased with the increase in the accumulation of heavy metals in fish organs. The induction in POD activity was noted with the stimulation of stress level along with the inhibition of CAT activity as well (Manoj and Padhy, 2013). At small concentration levels, induction of enzyme activity decreased as metal concentration increased (Shen *et al.*, 2009).

CONCLUSIONS

This study reveals the bioaccumulation of the metals and significant variations in antioxidant defense system of *Cirrhina mrigala* sampled from Indus River. Enzymes can serve as biomarkers for early detection of pollution during bio monitoring programs. Further studies on biomarkers

and bioaccumulation patterns in aquatic organisms will serve as a better tool for monitoring environmental pollution and improving ecological risk assessment associated with metals in aquatic ecosystems.

Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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