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Lead Induced Oxidative Stress in *Cirrhina mrigala*

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ABSTRACT

Lead (Pb) is a widely used metal in various industrial processes and is very persistent in the aquatic environment. The main objective of this study was to assess the time and dose dependent effects of Pb on bioaccumulation and oxidative stress in the liver, kidney, gills, muscles and brain of *Cirrhina mrigala*. The 120-days old fish were exposed to 1/4th (11.81mgL^{-1}) and 2/3rd (31.49mgL^{-1}) of 96-h LC_{50} of water-borne Pb for three fortnights (42 days) in glass aquaria at pH 7.5, temperature 30°C and total hardness 250mgL^{-1}. After each fortnight, fish were sampled from each treatment and different organs such as liver, kidney, gills muscles and brain were taken out for determination of bioaccumulation and oxidative stress in the fish. The results showed that water-borne Pb exposure caused significant accumulation in the fish tissues that increased significantly with increasing exposure of Pb. Pb accumulated variably in the fish tissues as liver > gills > brain > kidney > muscles. Oxidative stress in the Pb exposed fish was determined in terms of change in the activities of antioxidant enzymes viz. superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). The Pb exposure caused a significant time and dose dependent increase in the SOD and POD activities in selected tissues of *C. mrigala* while a significant decrease in the CAT activity was also observed. Liver showed a significantly higher activity of all the three enzymes that could be due to its actively metabolic nature. Pb can inhibit the functional properties of CAT by disrupting disulfide bond. Moreover, the interference of Pb in the activity of SOD was also reported during present investigation. Therefore, this study provides an insight in rational use of antioxidant enzymes as biomarkers of oxidative stress in the bio-monitoring of aquatic pollution.

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Authors' Contribution
MJ conceived the presented idea and supervised the work. FL performed the experimental work and wrote the paper. HAK and KR helped in the manuscript prepration and data analysis.

Key words
Bioaccumulation, Antioxidant Enzymes, Superoxide dismutase, Catalase

INTRODUCTION

Industrialization is vital to the socio-economic development of any nation as well as its standing in the international community. With its undeniable importance, there come the environmental issues that are associated with industrial activities. Due to the emergence of 4th industrial revolution, there is no escaping exposure to metals and their compounds because of increased dependence of our society on metallurgy for the proper functioning of a number of processes (Tan, 2017). Therefore, it is not surprising that human exposure to metallic ions has increased dramatically in the last few decades due to an exponential increase in the use of metal compounds in the industry and agriculture (Ezejiofor *et al*., 2013). Out of 388 cities of Pakistan, only 8 have wastewater treatment plants that can treat about 8% of domestic and industrial wastewater before disposal, however, the actual estimate is around 1% due to non-functioning of most of these treatment plants (Murtaza and Zia, 2012). According to gross operating profit (GOP) figures, 5.6 million tons of metal-based fertilizers and 10 thousand tons of pesticides are annually applied in agricultural lands of Pakistan (Daud *et al*., 2017). These chemical contaminants leach into the groundwater and enter in the lakes and rivers through agricultural runoff from where they get absorbed by finer particles and sink to the bottom. Exposure of aquatic biota, particularly fish to chemically contaminated waters pose serious threats to humans through the food chain (Ardeshir *et al*., 2017).

Lead (Pb) is a common, ubiquitous and persistent environmental pollutant with its increased worldwide production of about 2.5 million tons per year (Osfor *et al*., 2010). It interferes with a variety of physiologic and metabolic functions in the fish thus causing severe toxicity even at slight exposure (Jaishankar *et al*., 2014). It is not a transition metal hence, cannot readily undergo valence changes as chromium or copper metals. Pb can induce oxidative stress by directly affecting the cell membranes, interacting with hemoglobin and causes auto-oxidation of aminolevulinic acid or through the formation of complexes with selenium that decrease the antioxidant enzyme activity (Sevcikova *et al*., 2011).

During all toxicological processes in the fish, metals

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cross biological barriers directly through the skin and gills or indirectly through the walls of the digestive tract and accumulated in the metabolically active organs such as gills, liver, brain, and kidney (Firat *et al*., 2009a). Assessment of metals accumulation reflects the current health status of the fish before toxicity affect the ecological balance of the whole aquatic environment. Bioaccumulation of metals is frequently being used as a potential biomarker in toxicological studies (Birungi *et al*., 2007). In natural aquatic ecosystems, the toxic metal cations are relatively at sub-lethal levels that cause long-term multiple changes in the internal dynamics of aquatic fauna particularly fish (Firat *et al*., 2009b). However, various aquatic organisms are able to live in such contaminated waters due to their inducible defense processes. Free radicals are normally produced due to the cellular metabolism out of which mitochondrial respiration is the main endogenous source of reactive oxygen species (ROS). The normal process of ROS production is enhanced by various contaminants such as pesticides, metallic ions or petroleum products (Lushchak, 2011). Oxidative stress arises due to an imbalance between production and elimination of ROS by antioxidants (Birnie-Gauvin *et al*., 2017). Antioxidant enzymes viz. glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione-S-transferase (GST), peroxidase (POD) and catalase (CAT) are important indicators of oxidative stress (Pizzino *et al*., 2017). In the present study, *C. mrigala* were exposed to two different sub-lethal concentrations of water-borne Pb for three fortnights and the toxic effects of Pb to the fish were examined in terms of bioaccumulation and oxidative stress in the tissues such as liver, kidney, gills, muscles, and brain of the fish.

MATERIALS AND METHODS

Fish and experimental design

The 120-day old *Cirrhina mrigala* were obtained from the fish rearing earthen ponds of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan and transferred to the laboratory where they were acclimatized for 15 days prior to the experiments. After acclimatization, fish weighing approximately 15g were randomly divided into three groups with three replicate aquaria (50-liter water capacity). Each tank was stocked with 10 fish in 35L dechlorinated tap water at constant pH (7.5), total hardness (250mgL^{-1}) and temperature (30ºC). During these experimental trials, fish were fed with the supplementary diet containing 30% digestible protein and 3.0KCalg^{-1} of digestible energy. The fish were exposed to 1/4th (11.81mgL^{-1}) and 2/3rd (31.49mgL^{-1}) of 96-hr LC_{50} (47.23mgL^{-1}) of water-borne lead chloride ($PbCl_2$; Merck) which was already determined in our previous work.

Tissue sampling and Pb determination

During this experimental trial, after each fortnight three fish from each treatment were randomly sampled and dissected to isolate liver, kidney, gills, muscles, and brain, then washed with cold phosphate buffer and preserved for the determination of Pb and oxidative stress. Tissue samples were randomly selected from each replicate tank in every group. Each tissue sample weighed 1.5-2g and was obtained from two fish. These samples were then digested through a di-acid method in which nitric acid and perchloric acid were used by following S.M.E.W.W. (2012). The resulted solution was filtered and diluted up to 25ml with distilled water. Pb concentration was determined by atomic absorption spectrophotometer (AAnalyst-400, Perkin Elmer, USA).

Oxidative stress assays

Isolated samples of liver, kidney, gills, muscles, and brain were homogenized separately in cold phosphate buffer (0.2M, pH 6.5) and centrifuged at 10,000 rpm for 15 minutes at 4ºC and final supernatant was divided into sub-samples for SOD, POD and CAT assays. The SOD activity was measured by following the protocol described by Worthington (1988) with slight modifications and was described by its capacity to inhibit the photoreduction of nitro blue tetrazolium. The CAT activity was measured by its ability to reduce H_2O_2 at 240nm. It was calculated by following the protocol described by Chance and Maehly (1955), whereas POD activity was determined by measuring its capacity to reduce H_2O_2 at 470nm (Zia *et al*., 2011).

Statistical analyses

The resulted data were expressed as means±SD and subjected to two-way analysis of variance (ANOVA) by using Statistix$^{8.1}$ software to determine significant differences among them at $p<0.05$ significance level whereas means were compared by using least significant difference multiple range test.

RESULT

Lead bioaccumulation in the fish tissues

Table I shows that after exposure to water-borne Pb for three fortnights, *C. mrigala* exhibited a significant increase in Pb accumulation in all the sampled organs when compared to the fish in the control group. It was observed that the accumulation of Pb was significantly higher in fish exposed to 2/3rd of LC_{50} than that of 1/4th of 96-hr LC_{50}. Results also depict that Pb accumulation increases concomitantly with increasing exposure duration from 1st to 3rd fortnight. Furthermore, water-borne Pb

Table I. Time and dose dependent accumulation of Pb (μgg^{-1}) in the fish tissues during chronic exposure.

Duration	Treatments	Muscles	Kidney	Liver	Gills	Brain
	Control	0.81±0.21	2.46±0.11	5.94±0.04	5.87±0.02	2.55±0.12
1st fortnight	1/4th	9.52±0.04	15.46±0.03	25.54±0.22	23.87±0.27	19.24±0.11
	2/3rd	17.07±0.08	24.96±0.09	39.09±0.29	36.52±0.31	30.24±0.15
	Control	0.85±0.04	2.54±0.02	6.03±0.05	5.89±0.07	2.54±0.01
2nd fortnight	1/4th	16.83±0.07	26.85±0.10	37.96±0.27	36.62±0.32	31.29±0.16
	2/3rd	27.98±0.10	40.85±0.15	56.91±0.34	54.07±0.38	47.39±0.20
	Control	0.93±0.01	2.70±0.02	6.40±0.07	5.97±0.06	2.62±0.03
3rd fortnight	1/4th	28.57±0.11	47.39±0.19	63.27±0.36	59.32±0.42	53.92±0.21
	2/3rd	45.32±0.15	67.54±0.33	88.07±0.43	83.82±0.51	76.92±0.31

Table II. Time and dose dependent change in superoxide dismutase (UmL^{-1}) activity induced by Pb exposure.

Duration	Treatment	Muscles	Kidney	Liver	Gills	Brain
	Control	19.36±0.22	47.32±0.12	51.20±0.56	47.77±0.11	51.28±0.32
1st fortnight	1/4th	44.54±0.43	69.65±0.22	87.44±0.21	76.49±0.43	59.85±0.11
	2/3rd	64.22±0.32	96.87±0.31	125.74±0.63	114.23±0.32	76.25±0.24
	Control	34.77±0.27	68.54±0.19	64.49±0.11	52.74±0.21	46.55±0.43
2nd fortnight	1/4th	67.29±0.33	98.57±0.41	108.83±0.23	102.53±0.35	87.66±0.34
	2/3rd	104.74±0.38	135.48±0.54	142.55±0.33	136.29±0.22	116.86±0.52
	Control	45.82±0.20	59.77±0.22	75.33±0.24	65.22±0.21	51.67±0.53
3rd fortnight	1/4th	90.09±0.31	109.83±0.43	143.53±0.22	114.22±0.43	104.63±0.31
	2/3rd	127.54±0.52	156.82±0.47	170.52±0.54	146.39±0.27	142.96±0.44

exposure resulted in Pb accumulation in the fish tissues in the following order: liver > gills > brain > kidney > muscles (Table I).

Oxidative stress in the fish tissues

The chronic exposure of Pb to *C. mrigala* caused significant oxidative stress which was determined in terms of change in the activities of SOD, CAT, and POD. The SOD activity increased significantly with increasing Pb exposure concentration and duration (Table II). The sampled tissues exhibited significant variations in SOD activity as the maximum level of this enzyme was observed in the liver, followed by gills, kidney, brain, and muscles, however, during third-fortnight gills and kidney showed non-significant differences in SOD activity. Table III shows a significant decrease in the CAT activity with increasing Pb exposure duration in the selected tissues of *C. mrigala*. The CAT activity in the Pb exposed fish was significantly lower as compared to the control fish tissues. Among fish tissues, the CAT activity varied significantly as liver > gills > brain > kidney > muscles (Table III). Compared with the control group, water-borne Pb exposure resulted in a significant increase in POD activity in all the selected tissues at all exposure durations viz. three fortnights (Table IV). Moreover, the generation of POD increased gradually in exposure duration and dose-dependent manner in the tissues of *C. mrigala* that was significantly higher in the liver while it was significantly lower in the muscles (Table IV).

Relationship of Pb accumulation and oxidative stress

Table V shows the regression equations computed to determine the dependence of antioxidant enzymes activities on the accumulation of lead in various tissues of *C. mrigala*. Among three enzymes, SOD exhibited significantly positive dependence on the lead accumulation in all the tissues with the higher correlation coefficient ranging from 0.936 – 0.976. However, CAT activity showed highly significant but negative dependence on the lead accumulation in the muscles, kidney, liver, gills and brain of *C. mrigala*. The POD activity increased concomitantly with the increase in accumulation of lead as depicted by the higher coefficient of determination (R^2).

Table III. Time and dose dependent change in catalase activity (UmL^{-1}) induced by Pb exposure.

Duration	Treatment	Muscles	Kidney	Liver	Gills	Brain
	Control	698.81±1.33	761.73±1.56	797.78±1.78	781.62±1.65	763.36±1.58
1st fortnight	1/4th	553.03±1.25	680.72±1.48	764.99±1.75	740.78±1.62	710.17±1.51
	2/3rd	477.67±1.19	636.21±1.44	735.98±1.70	682.93±1.52	644.22±1.45
	Control	690.85±1.34	750.77±1.45	786.12±1.56	765.14±1.21	743.82±1.54
2nd fortnight	1/4th	532.90±1.66	654.33±1.62	727.65±1.43	708.64±1.55	670.52±1.23
	2/3rd	450.62±1.15	577.27±1.41	671.62±1.63	645.45±1.49	610.73±1.41
	Control	672.72±1.23	724.12±1.64	761.23±1.54	736.63±1.46	727.27±1.33
3rd fortnight	1/4th	476.98±1.44	629.24±1.56	678.72±1.42	643.92±1.43	640.82±1.52
	2/3rd	427.43±1.53	513.30±1.24	631.93±1.22	565.34±1.54	556.27±1.63

Table IV. Time and dose dependent change in peroxidase activity (UmL^{-1}) induced by Pb exposure.

Duration	Treatments	Muscles	Kidney	Liver	Gills	Brain
	Control	0.009±0.023	0.147±0.054	0.172±0.022	0.129±0.024	0.112±0.012
1st fortnight	1/4th	0.059±0.032	0.201±0.036	0.224±0.032	0.174±0.050	0.152±0.035
	2/3rd	0.131±0.012	0.285±0.043	0.305±0.044	0.249±0.021	0.222±0.028
	Control	0.037±0.041	0.216±0.042	0.267±0.021	0.185±0.028	0.144±0.034
2nd fortnight	1/4th	0.079±0.032	0.276±0.012	0.317±0.032	0.231±0.044	0.184±0.040
	2/3rd	0.140±0.022	0.365±0.051	0.399±0.043	0.306±0.031	0.258±0.024
	Control	0.054±0.042	0.293±0.030	0.360±0.033	0.226±0.042	0.148±0.032
3rd fortnight	1/4th	0.094±0.035	0.348±0.053	0.410±0.054	0.286±0.023	0.193±0.012
	2/3rd	0.164±0.022	0.434±0.020	0.490±0.022	0.376±0.044	0.269±0.024

DISCUSSION

Bioaccumulation of metals exhibits the quantity of pollutants/toxicant absorbed by the organism, the pattern of distribution among various tissues and the extent of which that particular toxicant accumulated in each of these tissues (Murugan *et al*., 2008). Knowledge of the metals distribution in tissues is quite useful in identifying the organs that are selective and sensitive to a particular metal's storage (Gbem *et al*., 2001). During present investigation, chronic exposure to Pb caused dose-dependent accumulation of Pb that also increased concomitantly with increasing exposure duration. Sen and Karaytug (2017) also reported exposure time and dose-dependent accumulation of Pb in the organs of *Oreochromis niloticus* and observed that maximum Pb accumulation was in brain followed by liver, gills, and muscles. Among the selected organs, liver accumulated significantly higher quantity of Pb that could be explained by the greater tendency of metals to react with the amino groups, nitrogen or sulphur groups of metallothioneins (metal binding proteins) that are in highest concentration in the liver (Al-Yousuf *et al*., 2000; Aich *et al*., 2012). Fish gills are multifunctional organs that perform various vital functions including osmoregulation, respiration, acid-base balance and excretion of nitrogenous waste (Oliveira-Filho *et al*., 2013). Furthermore, gill surfaces act as metal binding sites and enhance the metal's accumulation by facilitating the binding of positively charged metal ions to negatively charged ions on gills (Niyogi *et al*., 2015), whereas fish muscles exhibited significantly least tendency to accumulate Pb due to the lower concentration of metal binding proteins (Lemus *et al*., 2013). The brain of *C. mrigala* also accumulated a significant amount of Pb that can be attributed to the fact that by passing the blood-brain barrier, Pb replaces calcium ions in brain cells and get accumulated there (Nava-Ruiz *et al*., 2012). Das *et al*. (2015) reported the effects of sub-lethal concentrations exposure of Pb in the soft tissues of *Macrognathus pancalus*. After different exposure durations (3-42 days), the accumulation profile of Pb was brain > liver > kidney > gills > muscles > skin. In other words, the amount of a metal accumulated is influenced

Table V. Relationship between overall accumulation and antioxidant enzyme activities in the tissues of fish exposed to Pb.

Enzymes	Organs	Regression Equation for (y = a + bx)	r	R^2
		y= Enzyme Activity, x= Pb Accumulation		
SOD	Muscles	SOD Activity = 23.64 + 1.236 (Accumulation) $0.130^{(p<0.01)}$	0.958	0.917
	Kidney	SOD Activity = 49.78 + 1.199 (Accumulation) $0.140^{(p<0.01)}$	0.949	0.901
	Liver	SOD Activity = 63.47 + 1.727 (Accumulation) $0.174^{(p<0.01)}$	0.961	0.924
	Gills	SOD Activity = 58.63 + 2.219 (Accumulation) $0.285^{(p<0.01)}$	0.939	0.882
	Brain	SOD Activity = 44.45 + 1.266 (Accumulation) $0.101^{(p<0.01)}$	0.976	0.952
CAT	Muscles	CAT Activity = 679.4 - 3.633 (Accumulation) $0.566^{(p<0.01)}$	- 0.913	0.834
	Kidney	CAT Activity = 758.0 - 2.717 (Accumulation) $0.278^{(p<0.01)}$	- 0.960	0.922
	Liver	CAT Activity = 790.40 - 2.417 (Accumulation) $0.212^{(p<0.01)}$	- 0.970	0.942
	Gills	CAT Activity = 769.26 - 4.415 (Accumulation) $0.345^{(p<0.01)}$	- 0.976	0.953
	Brain	CAT Activity = 748.3 - 2.504 (Accumulation) $0.266^{(p<0.01)}$	- 0.957	0.917
POD	Muscles	POD Activity = 0.0276 + 0.001663 (Accumulation) $0.00031^{(p<0.01)}$	0.867	0.752
	Kidney	POD Activity = 0.1866 + 0.002689 (Accumulation) $0.000570^{(p<0.01)}$	0.853	0.727
	Liver	POD Activity = 0.2347 + 0.003604 (Accumulation) $0.000916^{(p<0.01)}$	0.802	0.644
	Gills	POD Activity = 0.1662 + 0.004504 (Accumulation) $0.000710^{(p<0.01)}$	0.911	0.831
	Brain	POD Activity = 0.1330 + 0.001819 (Accumulation) $0.000370^{(p<0.01)}$	0.862	0.744

by various environmental, biological and genetic factors, leading to the differences in metal accumulation between different individuals, species, age, tissues, seasons and sites. Aquatic pollution is a major contributor to oxidative stress in the fish that results from oxidation and redox cycling of pollutants. Oxidative stress can be induced by an increase in the production of ROS or incapacity of antioxidants to repair oxidative damage (Dorval and Hontela, 2003). Among the antioxidant enzymes, SOD is the first enzyme in the frontline defense in detoxification of superoxide radicals by dismutating them into molecular oxygen and hydrogen peroxide. Furthermore, CAT and POD convert the hydrogen peroxide into water and oxygen (Ates *et al*., 2008). Metals are well-known inducers of oxidative stress and the investigation of this stress level in the fish is beneficial to estimate the responses of antioxidants against metals since intrinsic factors viz. diet type, feeding behavior, phylogenetic position and oxygen consumption affects directly the enzymes responses (Martinez-Alvarez *et al*., 2005). Redox-active metals produce ROS through redox cycling, whereas redox-inactive metals impair antioxidant enzymes defense, especially that of thiol-containing enzymes. Lead being a redox-inactive metal, causes the alteration of hematologic systems of fish by inhibiting the enzyme activities involved in heme biosynthesis. During present investigation, Pb exposure causes an increase in SOD activity in all the tissues of *C. mrigala*. The induction of SOD and CAT at the same time has been reported in several teleosts after exposure to metallic ions (Livingstone, 2001; Pandey *et al*., 2003; Fernandes *et al*., 2008). It is a common mechanism of adaptation in response to the oxidative stress in the fish that are tissue specific (Stephensen *et al*., 2002). Ultimately, these enzymes take significant part in the maintenance of comparatively low levels of reactive species in the cells (Hidalgo *et al*., 2002). The CAT activity decreased significantly due to Pb exposure as compared to control that can be attributed to the fact that reaction of lipid peroxidation products with amino acids of antioxidant enzymes can change normal protein functioning hence, causing a decreased enzyme activity as well (Bagnyukova *et al*., 2006). Moreover, the carbonylation of proteins can also reduce the activity of the antioxidant enzyme. The decline in the activity of CAT may be due to the increased activities of complementary SOD and POD (Oruc and Usta, 2007). Lead caused a significant decrease in CAT activity by interfering with heme synthesis and forming a complex with selenium (Manoj and Padhy, 2013). Since the toxicants concentration and exposure duration, as well as functional ability of the tissues, determine the type of antioxidants mechanisms, the use of antioxidant enzymes responses in ecological and toxicological studies are of highly toxicologically relevant and an important index of environmental health status (Atli and Canli, 2007).

CONCLUSION

Present investigation revealed variable toxicity of Pb to the *C. mrigala* in terms of bioaccumulation and oxidative stress in different fish tissues. All the fish tissues showed significantly variable exposure dose and time-dependent accumulation of Pb that followed the order: liver > gills > kidney > brain > muscles. The Pb exposure caused oxidative stress in the fish that was depicted by the increase/decrease in antioxidant enzymes activities when compared to control. Lead exposure caused a significant time and dose-dependent increase in the SOD and POD activities in selected tissues of *C. mrigala* while a significant decrease in the CAT activity was also observed. This study provides an insight in the rational use of antioxidant enzymes as biomarkers of oxidative stress in the bio-monitoring of aquatic pollution.

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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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