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Species specific stress and anti-oxidant activity against metal induced toxicity in Indian Major Carps

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Abstract (12pt)

Concentrations of different metals (Cu, Ni, Zn, Cd, Pb) were measured in sediment, water, and gill tissue of three Indian Major Carps (*Labeo rohita, Catla catla, Cirrhinus cirrhosus*) collected from ponds at two different sites (Nalban Bheri and Diamond Harbour). Gill tissues were analyzed for the levels of different antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRd), glutathione-S-transferase (GST), glutathione (GSH) and malondialdehyde (MDA). Concentration of all metals were significantly higher ($P<0.05$) in sediment, water and gill tissue from Nalban Bheri compared to that in Diamond Harbour. Activities of all enzymatic and non-enzymatic antioxidant parameters were significantly higher ($P<0.05$), while $Na^+ K^+$ATPase level reduced significantly ($P<0.05$) in gill tissue from Nalban Bheri compared to those in Diamond Harbour. Significant multicollinearity was found in the values of SOD, CAT, GST, GRd, GPx and MDA with Pb, Cu and Ni in all three fish species at Nalban and with Cd in *L. rohita* and *C. catla*. Principal component analysis results revealed that stress response in polluted site was directly regulated by combination of GSH profile and the levels of MDA in a synchronized manner. The study indicated species-specific difference for metal induced oxidative stress response in fish gill and a correlation between different metals and individual oxidative stress markers.

Keywords:

Metal toxicity .
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Gill,
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Oxidative stress
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1. Introduction

Pollution of aquatic environment with different metals has become a great concern in recent years because of their non-biodegradable nature, long biological half-life and their potential to accumulate in different body parts of organism (Kim *et al*., 2011). The toxicity of such metals might vary greatly between organisms for the same metals, and between metals for the same organisms. Furthermore, metals might not necessarily follow the same rank order of toxicities between organisms, depending on differences between uptake rate, detoxification rates and excretion rate of the different organisms compared (Luoma and Rainbow, 2008). At higher levels of biological organization such metals may induce changes in metabolism, biochemistry, physiology, histology, inhibit synthesis of proteins and nucleic acids, and thus act as major stress factors to aquatic organisms (Seebaugh et al., 2005; Bertin and Averbeck, 2006). Essential metals such as copper (Cu), nickel (Ni) and zinc (Zn) has important biological roles, and toxicity occurs at high concentrations, while nonessential metals such as cadmium (Cd) and lead (Pb) are toxic to living organisms at very low concentrations (Dogan et al., 2015). Cadmium is a non essential, non-biodegradable element reported to be a major contaminant that causes adverse effects on the aquatic system, Lead pollutant induces lipid peroxidation in tissues and causes an irreversible damage to the respiratory organs of fish, and nickel induces a morphological transformation and chromosomal aberration in cells (Authman et al., 2015).

Fish are regarded as indicators of metals contamination in the aquatic ecosystem as they occupy high trophic levels and are important food source (Agah et al., 2009). Metals may enter fish through different routes such as via food or non-food particles, gills, oral consumption of water and the skin (Ay et al., 2009). On absorption, the pollutant is carried in blood stream to either a storage point or to the tissue for transformation and/or storage. Pollutants transformed in the liver may be stored there or excreted in bile or transported to other excretory organs such as gills or kidneys for elimination or stored in fat, which is an extra hepatic tissue (Heath, 1991; Nussev, 2000). Several studies have reported the distribution of metals in different organs like the muscles, liver, kidneys, heart, gonads, bone, digestive tract and brain of fish. Moreover, metal bioaccumulation by fish and subsequent distribution in organs is often regarded to be greatly inter-specific (El-Moselhy et al., 2014).

Any change in the natural conditions of aquatic medium causes several physiological adjustments in fish. Several studies have demonstrated that metals can promote the formation of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (-OH) in aquatic organisms including fish, because of oxidative metabolism (Kim *et al*., 2011). The hydroxyl radicals can initiate lipid peroxidation (LPO) in tissues. To attenuate the negative effects of ROS, fish possess an antioxidant defense system that utilizes enzymatic and non-enzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRd) and glutathione-S-transferase (GST). The non-enzymatic defense includes Vitamin–E, C, and A, glutathione (GSH), carotenes and ubiquinol10 (Filho, 1996). These oxidative stress biomarkers are often used in ecotoxicology to provide an early warning of potentially damaging changes in stressed fish (Pandey et al., 2003).

Indian major carps (*Labeo rohita*, *Catla catla* and *Cirrhinus cirrhosus*) are important commercial species in the Indian subcontinent that caters to large populations. These are often cultured in polluted waters and thus susceptible to exposure to different heavy metals. The fish must exhibit adaptive response to thrive in a polluted aquatic environment. However, little information is available regarding the effects of metals on oxidative stress response in gills of the three Indian major carp species. In the present study, levels of metals in gill of three Indian Major Carps from two different collection sites were determined, aiming to evaluate the environmental status of the water bodies used for aquaculture. Moreover, the levels of different oxidative stress biomarkers were measured in gill tissue of *L. rohita*, *C. catla* and *C. cirrhosus* to determine any species-specific difference for oxidative stress response among Indian major carps. Efforts were also made to determine correlation, if any, between different metals and individual oxidative stress markers to predict the most important biomarker for specific metal contamination.

2.Materials and Methodologies

Selection of site

One pond each from Nalban Bheri, Kolkata (22°56'N; 88°43'E) and Diamond Harbour, South 24 Parganas (22°19'N; 88°18'E) were selected as study sites. Nalban Bheri is located within East Kolkata wetland and the water is contaminated with domestic and industrial effluents from the city. This water body is extensively used for production of Indian major carps through sewage fed aquaculture. Carps are also cultured at the selected water body in Diamond Harbour, which mainly receives effluents from rural domestic sewage.

Collection of water and sediment samples

Surface water and sediment samples were collected randomly from 5 different points through the banks of the selected pond at both study sites. Water was collected at a depth of 1m using clean stainless steel buckets,

filtered, acidified to a pH of <2.0 with concentrated nitric acid (HNO_3), transported to the laboratory and stored at 4^0C until further analysis. Sediment samples were collected using a Van Veen grab sediment sampler, packed in clean polythene bags and stored.

Collection of fish tissue samples

Three Indian major carps, *L. rohita*, *C. catla* and *C. cirrhosus*, were collected from the same pond at two study sites from where water samples were procured (fish weight~500 g) (n=6, for each species), preserved immediately in ice and brought to laboratory. In the laboratory, fish were dissected to obtain gill tissues from each fish. Gill samples were stored at -20^0C until further analysis.

Estimation of metals in water and sediment

Concentrations of metals such as Pb, Cd, Cu, Ni and Zn in water and sediment samples were determined by standard methods following APHA (1998) and Nafde *et al*. (1998), respectively. In brief, the stored water samples were further acidified with 5ml/l conc. HNO_3. Thereafter, 100 ml of the samples were taken, 5ml of high purity HCl was added to it, and the sample was heated for 15 minutes in fume bath. The samples were then cooled, filtered by Millipore filter paper and the filtrates were digested by a proportionate mixture of concentrated HNO_3-$HClO_4$ and finally filtered in Watmann filter paper (grade-42) before detection of heavy metal concentrations using atomic absorption spectrophotometer (AAS, Thermo Scientific ICE-3000 Series). Sediment samples were air dried, ground to a fine powder, and then passed through a plastic sieve (100 mesh size). Sieved sediment samples were acidified overnight with 5 ml/g conc. HNO_3 at room temperature and thereafter processed similarly like water samples for detection of metal concentration in AAS.

Estimation of metals in fish gill tissues

A modified wet digestion procedure was used to prepare biological samples for the determination of aforementioned metals in AAS (Chernoff, 1975). In brief, 1 g of gill tissue was kept for overnight digestion in 5 ml of concentrated HNO_3. The samples were then filtered in Watmann grade 1 filter paper and the filtrate was diluted to 100 ml in volumetric flask and 5 ml conc. HCl was added and the mixture was placed in fume bath for 15 minutes. After that the samples were cooled, filtered by Millipore filter paper and the filtrates were digested by a proportionate mixture of concentrated HNO_3-$HClO_4$ and finally filtered in Watmann filter paper (grade-42) before detection of metal concentration in AAS.

Tissue sample analysis for anti-oxidative study

A part of the gill tissue was stored in ice cold phosphate buffer until it was homogenized and sonicated at 4°C in a homogenizing buffer (50 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, 100 mM sucrose, 1 mM PMSF, and 1% leupeptinhemisulphate), to prepare 10% tissue homogenate, which was stored at -80ºC until used for further study.

All enzymatic and non-enzymatic parameters of oxidative stress such as SOD, CAT, GPx, GRd GST, GSH and MDA were estimated according to procedures mentioned in Das *et al*. 2017.

Na^+K^+ATPase analysis

Na+/K+-ATPase activity in gill tissue was measured using the standard protocol mentioned in Piermarini and Evans, 2000.

Data Analysis

Concentration of metals, Na^+K^+ATPase leveland the antioxidant enzyme parameters in gill tissues of fish from two sites were analyzed by Univariate ANOVA. The main factor effects means were compared with Fisher's LSD test (at 5%) (Fisher, 1935), if found significant. The statistical works were performed using *XL*STAT software following Scheffe (1999). All data were expressed as mean ± standard deviation (SD).

Six separate principal component analysis (PCA) for all the antioxidant enzymes with respect to metal concentrations were done to understand the ordination. First, interspecific patterns of stress were examined using a PCA on the correlation matrix of standardized enzymes and metals. This analysis allows comparison of stress physiology in respect to metallic deposition in gills within a multivariate morphological space and identification of patterns of correlation among physiological variables. Eigen values of each component of the PCA were considered to interpret results.

3. Results

The concentration of all the five metals in sediment and water of the pond at Nalban Bheri was found to be significantly higher ($P<0.05$) compared to those in the pond at Diamond Harbour (Table 1). The concentration of Zn was found to be the highest in sediment and water at both the sampling sites. Concentrations of Pb and Cu were found to be higher in sediments than those in water from both study sites. Ni concentration was negligible in the sediment and water at Diamond Harbour (Table 1).

The concentration of all five metals was observed to be significantly higher ($P<0.05$) in fish gill tissues from Nalban Bheri compared to those in Diamond Harbour (Fig. 1). At Nalban Bheri, Zn was found to be accumulated in the fish gill tissues at the highest concentration followed by Ni and Cd (Fig. 1). The concentrations of metals in gill tissues varied in different fish species. Zinc concentration was the highest in gills of *L. rohita*, while Ni concentration was the highest in gills of *C. catla* (Fig. 1). There was no significant

difference (P>0.05) in concentration of Cu and Pb between different fish species (Fig. 1). In Diamond Harbour, however, concentrations of Zn, Ni and Cd were found to be the highest in gills of *C. catla* (Fig. 1).

Table 1: Concentration (ppm) of five heavy metals in soil and water from ponds at two sampling sites.

Sample	Metal Concentration (ppm)				
	Pb	Cu	Cd	Ni	Zn
Nalban bheri sediment	18.95±1.08*	21.57±1.5*	4.42±0.66*	9.49±0.78	32.37±2.81*
Diamond Harbour sediment	2.32±0.62	2.48±0.49	0.34±0.04	BDL	2.87±0.36
Nalban bheri water	11.66±0.93#	5.69±0.42#	4.44±0.23#	3.99±0.01	22.75±1.97#
Diamond Harbour water	1.13±0.04	0.25±0.003	0.03±0.002	BDL	4.6±0.7

*Note: Data are means ± S.D. * denotes significant variation (P<0.05) in mean values of metals in sediment between two sites, # denotes significant variation (P<0.05) in mean values of metals in water between two sites. Pb: Lead, Cu: Copper, Cd: Cadmium, Ni: Nickel, Zn: Zinc. (BDL: Below Detection Level)*

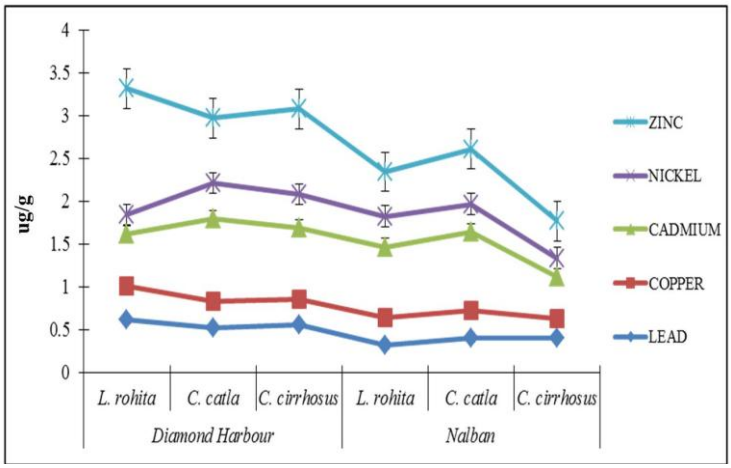
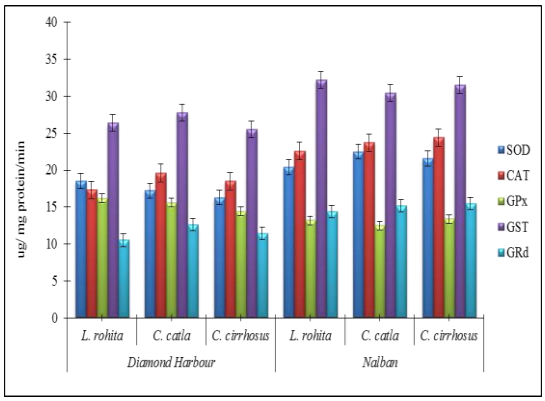
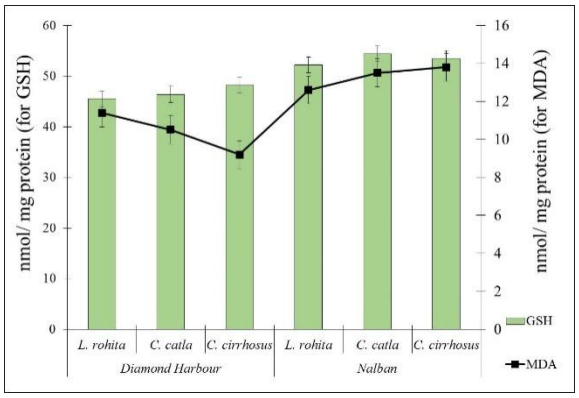


Figure 1- Profile of five metals in gill tissue of three IMCs collected from Nalban and Diamond Harbour.

The activities of all enzymatic and non-enzymatic antioxidant parameters except GPx and GRd were significantly higher (P<0.05) in tissue samples from Nalban Bheri compared to those in Diamond Harbour (Fig. 2). Activity of GPx was observed to be significantly lower (P<0.05) at Nalban Bheri than that at Diamond Harbour. There was significant difference (P<0.05) in the activities of SOD, CAT, GST and GSH among the three fish species collected from Nalban Bheri. Gill tissue from three fish species at Nalban Bheri showed significantly higher (P<0.05) SOD, CAT, GST and MDA activity compared to those at Diamond Harbour (Fig. 2). There was no significant difference (P>0.05) in GPx levels among three different fish species at both sites. GSH and MDA levels were found to be significantly higher in gill tissues from fish at Nalban Bheri compared to those at Diamond Harbour (Figure 2). At Nalban Bheri, the highest GSH level was observed in *C. catla*, while at Diamond Harbour, *C. cirrhosus* showed the highest GSH level (Fig. 2). However, MDA level was the highest in *C. cirrhosus* at Nalban Bheri and in *L. rohita* at Diamond Harbour ((Fig. 2).

Gill tissues from all fishes at Diamond Harbour showed significantly higher level (P<0.05) of Na^+K^+ATPase activity compared to those at Nalban Bheri (Fig. 3). No significant variation in Na^+K^+ATPase activity was

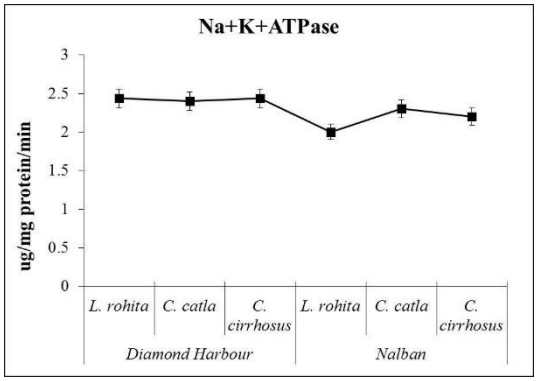




observed among the three different fish species in Diamond Harbour, while Na^+K^+ATPase activity in different fish species from Nalban Bheri showed significant interspecific variations (Fig. 3).

Figure 2- Profile of (a) enzymatic and (b) non-enzymatic antioxidants in gill tissue of three IMCs collected from Nalban and Diamond Harbour.

(a)



(b)

Figure 3: profile of Na^+K^+ATPase activity in gill tissue of IMCs collected from Nalban and Diamond Harbour

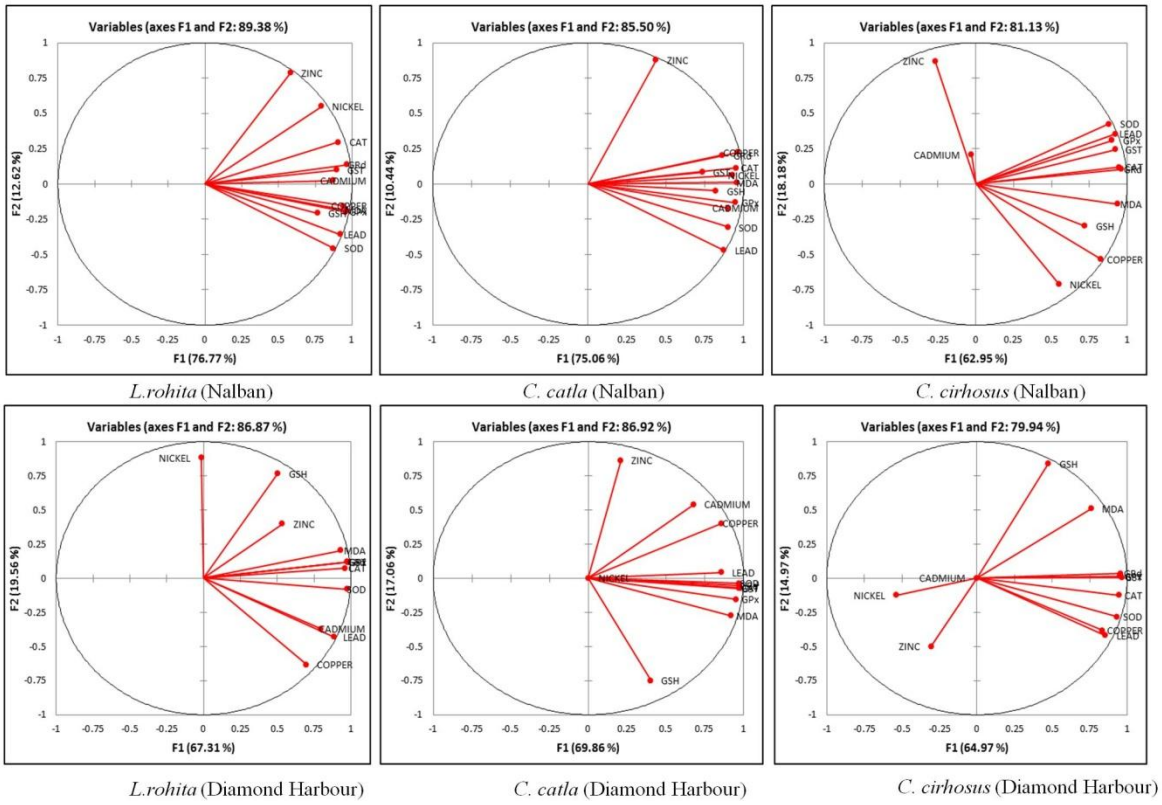


Figure 4- PCA of gill tissue IMCs collected from Nalban and Diamond Harbour showing relation of heavy metal and antioxidant

PCA replaces the original variables by a much smaller set of derived variables that embody as much as possible of the variance of the data, thereby aiding interpretation. Essentially the PCA takes a set of data points, and rotates it such that the maximum variability is visible, thus identifying the most important gradients. Considering the strength of the correlation coefficients between different physiological variables, a significant

amount of multicollinearity was found in the values of selective anti-oxidative enzymes (SOD, CAT, GST, GRd and GPx) and stress markers (MDA and GSH) with Pb at both sites for all three fish species(Fig. 4). No multicollinearity was observed between the antioxidant enzymes, stress markers and Zn (Fig. 4).

4. Discussion

The distribution processes of the metals that enter natural water are controlled by a dynamic set of physicochemical interactions. Sediments are the major repository of metals. However, metal concentration in the water becomes significantly higher due to allochthonous and autochthonous processes (Olowu et al., 2010). The important metals such as copper and zinc are added in the aquatic system from domestic sewage while agricultural and industrial runoffs contribute most of non essential metals (Ambedkar and Muniyan, 2011). High metal concentrations in both soil and water fractions of the pond at Nalban Bheri were due to the higher sewage input. Presence of metals at higher levels may be considered as environmental hazards as fish is especially susceptible to waterborne metals that are ultimately transferred to the human food chain (Giri and Singh, 2014). Similar order of abundance of metals as observed in the present study was reported in water and sediments of other sewage fed fish ponds at East Kolkata wetland (Kumar et al., 2010). The low level of contamination in pond at Diamond Harbour might be because of the presence of agricultural run-offs and ground water discharge due to water upwelling. Cu has higher affinity for organic particles. It binds maximally to the sediments and are lowly available as ions in the overlying water. Zinc and lead have intermediate affinity and hence occur in both water and sediment of ponds at two sites (Table 1). Cadmium was found to be distributed equally in both water and sediment of pond at Nalban Bheri. Considering the accumulation pattern of metals in ponds at two sites, it may be inferred that the pond at Nalban Bheri is more polluted compared to the pond at Diamond Harbour.

Metal accumulation in gills of different fish species varies due to differential metabolic demand between the species. Moreover, various environmental factors, feeding habit, habitat, age, sex and body weight of fish play key role in pattern of metal accumulation (Authman, 2008). Several studies have indicated species-dependent bioaccumulation pattern of metals as observed in the present study (El-Moselhyet al., 2014; Malik

et al., 2014). The relative rates of metal binding and release determine the metal accumulation in various tissues. The uptake and distribution of one metal can as well be altered by chronic sublethal exposure to another metal. Besides, accumulated elements are regularly released from the body as part of the homeostatic regulation of the organism. Metal accumulation in various organs often depends upon the ability of the fish species to regulate and excrete the contaminants. Gill has been found to accumulate maximum amount of metals such as Zn, Ni, Cu and Cd in many different fish species (Malik et al., 2014; Javed and Usmani, 2016). The production of the low molecular weight protein metallothionein in gill is reported to increase in response to elevated metal level. Metallothionein binds to the metals to detoxify them (Javed and Usmani, 2016).

Essential elements such as Cu and Zn are homeostatically regulated in the body. On the other hand, the nonessential metals accumulate in proportion to their ambient concentration. This sediment-associated metal was found to accumulate in comparatively lower amount than Zn (Fig. 1). Generally, Pb accumulates in tissues in proportion to the ambient concentration (Tao et al., 1999), but the rate of its depuration is dependent on the overall body concentration (Schulz-Balder, 1974).

The results of the present study showed that the activities of CAT, SOD and GST were affected most among all the selected carp caught from more polluted site (Nalban) compared to the less-polluted pond (Diamond Harbour). Such changes indicate the presence of oxidative stress in fishes from the more polluted site. Being the most important inducible antioxidant enzymes, the levels of these three enzymes increased with metal related oxidative stress. SOD catalyzes the conversion of superoxide radicals to H_2O_2 and O_2, and possibly the first enzyme to combat with generated oxy-radicals (Kappus, 1985). Enhanced levels of both SOD and CAT activity indicate the formation of the adaptation equilibrium (Di Giulio et al., 1989). Therefore, these two are responsible for the earliest adaptation response to oxidative stress and might be most potential biomarker of heavy metal toxicity.

Peroxisomal enzyme CAT converts excess H_2O_2 to H_2O and O_2; while GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides to H_2O and O_2 and is considered as an efficient protective enzyme against lipid peroxidation (Winston and Di Giulio, 1991). Earlier, CAT was already found to be induced in response to exposure to contaminated aquatic system in different fish species (Livingstone et al., 1993; Rodriguez-Ariza et al., 1993) and in marine mussels (Porte et al., 1991).

GRd maintain the cytosolic concentration of reduced glutathione, induction of which is a potential biochemical marker of oxidative stress (Stegeman et al., 1992). Decreased GRd activity might lead to GSH depletion. Therefore, regular adjustment must be needed by the synthesis of new glutathione molecules. Considering its importance in maintaining the antioxidant balance during xenobiotics toxicity, fish GRd has received much attention in biomonitoring studies. Earlier studies (Stegeman et al., 1992) have reported a significant increase in hepatic GRd activity after oxidative stress though the mechanism of regulation of GRd in fish is still not understood. The elevation in its activity can be interpreted as a secondary adaptive response to oxidative stress. The result of the present study is in accordance with such previous observation. GSH, the major cytosolic sulfhydryl compound, acts as a primary reducing agent and a protective marker against plentiful toxic substances including inorganic and metal pollutants, through its -SH group (Stryer, 1988). Hence, GSH is always the first line of defence against oxidative stress. GSH levels increase due to an adaptive mechanism to slight alteration in oxidative status. However, severe oxidative stress might suppress GSH levels due to loss of such adaptive machinery and the oxidation of GSH to oxidized glutathione (GSSG). Fish cells after contact with some pollutants, removed immediately through conjugation with GSH or by means of GSTs, which decreases the levels of GSH. In addition, the oxidative damage caused by metabolites of some inorganic and metal pollutants by uncoupling of mitochondrial oxidative phosphorylation (Fang, 1975), which would generate free radicals or ROS. The detoxification of ROS and hydroperoxides implies the oxidation of GSH to GSSG by the action of GPx. GSSG is then reduced back to GSH by GRd at the expenses of NADPH, which is recycled through the pentose phosphate pathway. Whenever the generation of GSSG becomes higher than its reduction back to GSH, GSSG accumulates and it is translocated outside the cell to avoid NADPH exhaustion (Kaplowitz et al., 1996; Keppler et al., 1997). Low GRd activity means the production of GSSG is low and GPx activity is also low. Consequently, this is followed by a depletion of the GSH pool (Eroglu et al., 2015). Our results indicate the crucial role of metals, to participate in several oxidation-reduction reactions, resulting in the formation of secondary components such as ROS (Charrier et al., 2014).

Immense antioxidant defense enhancement in the form of increased catalase activity in gill (Fig. 2) was observed in fish collected from the polluted area, the same response as that found in the mullet (Rodríguez-Ariza et al., 1993), but opposite to that found in the Nile tilapia (Bainy et al., 1996). A similar pattern was observed for GST activity which emphasize the need for proper physiology, irrespective of the stimulation of CAT and/or glutathione peroxidase, and that is important to keep constitutively high levels of reduced glutathione. The concentrations of total and reduced glutathione found in the gill of all the selected carp species were similar to those usually found in other fish species exposed to pollutants. Reduced glutathione

and MDA level concentrations in the carp showed an inverse correlation, and the similar relationship was found in other fish species (Park, 2015). Acute and subchronic exposure to pollutants enhances reduced glutathione contents in fish gill cells (Al-Ghais, 2013) while after severe and chronic exposure seems to be unable to maintain high constitutive total glutathione levels. A sharp decrease observed both in reduced and oxidized glutathione concentrations may be attributable to the decrease in total glutathione (Meng et al., 2015). The higher Na^+K^+ATPase activity in gills of fishes from Diamond Harbour indicates better physiological homeostatic condition in unpolluted environment.

Our PCA results revealed that stress response in polluted site was directly regulated by amalgamation of GSH profile and the levels of MDA in a synchronized manner and the synchronization was to some extent controlled mainly by both SOD and CAT with little contribution from other antioxidant enzymes, who act in a concert to remove reactive oxygen species created during metal-induced stress. However, at Diamond Harbour, GSH doesn't seem to be much correlated with any specific metal composition or any other antioxidant parameters indicating that in unpolluted system GSH might be regulated independently. This might be viewed as a decisive step towards the derivation of explanatory frameworks for prediction of site-specific pollutant impact on fish health, growth and reproduction. The immense advantage of such simulation is that it perhaps persuades an understanding of how all these processes are linked and how system properties emerge in space and time (Hunter et al., 2002). Analyzed data also indicated that all the biochemical and metal variables were highly correlated except zinc in all the selected carp and whereas cadmium also didn't seem to have much role in determining stress factors in *C. cirrhosus* which supports the fact that ecosystem-level functional properties and response to any specific stressor of the environment depends on the individual species health-status (Moore et al., 2006; Rees et al., 1999).

5. Conclusion

Our experiment revealed the relative change in stress physiology attributable to metal exposure. The magnitude of metal-induced stress that can be tolerated by an organism is a function of how well the animal is equipped to compensate for the effects of exposure. Our results indicate that Pb can induce higher toxic response in fish even at low concentration. On the other hand, Zn though present at the highest concentration in fish gills, generates minimum toxic response. Cumulative effects of multiple metal stressors can deplete an organism's reserves, thereby reducing its ability to cope. Conversely, organisms in relatively unpolluted environments may have large energetic reserves that can be mobilized to maintain homeostasis.

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