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BIOCHEMICAL CHANGES INDUCED BY CADMIUM, COPPER, LEAD AND ZINC EXPOSURE TO PERNA VIRIDIS UNDER LONGTERM TOXICITY TEST

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ABSTRACT

Environmental pollution is a growing concern and, more importantly, pollution of the aquatic ecosystem is alarming. Bivalves are commonly used as bio-indicators of marine pollution. In the present study the *Perna viridis* was exposed to cadmium, copper, lead and zinc concentrations under longterm chronic toxicity for the estimation of biochemical biomarkers. Significant differences in TBARS levels and antioxidant enzyme activity were found. Higher levels of TBARS were found with cadmium exposure. This study suggests that TBARS levels and antioxidant enzymes activity are affected by biotic and abiotic factors and have potential as indicators of heavy metal contamination. Since these results were also found to vary, the determination of oxidative stress biomarkers in *Perna viridis* may serve as a convenient approach during pollution biomonitoring programme.

KEYWORDS

Oxidative stress, antioxidants, reactive oxygen species, heavy metals, lipid peroxidation.

INTRODUCTION

Trace metals at the cellular level are often involved in oxidative stress, which results in the production of Reactive Oxygen Species (ROS). ROS includes the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical, all of which affect mainly lipids, proteins, carbohydrates, and nucleic acids[1]. The importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses by scavenging of ROS. The antioxidant system comprises several enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), and Guaiacol Peroxidase (GPx). Superoxide radicals that are generated are converted to H_2O_2 by the action of SOD, and the accumulation of H_2O_2 is prevented in the cell by CAT and GPx. It has been demonstrated that the activities of SOD, CAT, and GPx are induced upon metal exposure to animal species by some external factors[2]. Excessive ROS production in response to heavy metal pollution detoxification can overwhelm natural defense mechanisms leading to cumulative damage to biomolecules[3]. When the antioxidant enzymes fail or are insufficient, an increase of ROS production may originate oxidative damage[4].

Enzymatic antioxidants have great potential to indicate the toxic effects of heavy metals because they can generate free radicals, which cause lipid peroxidation of cell membranes[5]. Glutathione *S*-transferase (GST), a detoxification enzyme involving in the conjugation of xenobiotics with endogenous substrate, thus facilitates their excretion. GST appears to be sensitive to both pollution and natural factors, which can hinder the interpretation of the results[6]. Marine bivalves such as snails, oysters and mussels were widely used as bioindicators of contamination in the monitoring of pollutant effects. As filter feeders, these species are known to be good general indicators of chemical contamination[7].

Some heavy metals are required at structural and catalytic sites in protein and are regarded as essential metals; others do not have a known biological function and are thus considered as nonessential metals[8]. Cadmium and zinc, the typical nonessential and essential metals are often found together in the environment because of similar properties, but biologically they have diverse properties. Both of them can be toxic when present above the critical level[9]. Determinations of sulfhydryl group levels which metals have high affinity towards, and total protein levels could be beneficial in estimating the toxicity of metals[10].

The antioxidant defense system is being studied because of its potential utility to provide biochemical biomarkers that could be used in environmental monitoring system[11]. Biomarkers in environmental monitoring confer significant advantages over traditional chemical measurements because measured biological effects can be meaningfully linked to environmental consequences so that environmental concerns can be directly addressed[12]. Although the activity of antioxidant enzymes may be increased or inhibited under chemical stress, there is, however, no general rule for the different enzymes[13]. The antioxidant enzymes are often measured together to indicate the total oxyradical scavenging capacity and this has been observed to provide greater indicating value[14, 15]. Hence in the present study the responses of lipid peroxidation, glutathione *S*-transferase, catalase, reduced glutathione (GSH), acetylcholinesterase and total proteins were estimated in the tissues of *Perna viridis* exposed to cadmium, copper, lead and zinc under chronic toxicity test for 30 days.

MATERIALS AND METHODS

Juvenile specimens of *Perna viridis* (1.6 ±0.4cm in length and 0.12 ±0.01g) were immediately transported to the laboratory in air filled plastic bags and acclimatized in glass aquaria with aerated natural filtered seawater for a period of 8 days at 28 PSU salinity, temperature of 28 ±2 °C, dissolved oxygen of 5.6 mg/l and pH of 8.01. Stock solutions of cadmium, copper, lead and zinc were freshly prepared by dissolving the proper metal salts of cadmium chloride hemi (pentahydrate), copper (II) chloride, lead (II) nitrate, and zinc sulfate in deionized (double distilled) water. Fresh stock solutions were prepared daily. These solutions were serially diluted to get the experimental concentration for the toxicity test. The experimental method includes static renewal (24 hour renewal) test [16]. Five concentrations in a geometric series including control were prepared for the test for 30 days in chronic toxicity test [17]. Toxicant and seawater were replaced on daily basis. Test animals were fed three times during the test. Maximum-allowable control mortality was 20 per cent for 30 days for chronic [17].

SAMPLE PREPARATION

At the final stages of the chronic toxicity test, the tissue samples of survived test animals were pooled and made in duplicates. For the analysis of lipid peroxidation marker and antioxidant enzyme activities, 1g tissue was homogenized in chilled pestle and mortar with 5ml homogenization buffer (0.25M sucrose, 10 mM Tris, 1 mM EDTA, and pH 7.4) and centrifuged at 5,000 rpm for 15 minutes at 4°C. The resulting supernatant was the homogenate which was used for the estimation of various biochemical assays.

LIPID PEROXIDATION (LPO)

Lipid peroxidation level was assayed by measuring Malondialdehyde (MDA), a decomposed product of polyunsaturated fatty acids. Hydroperoxides were determined by the thiobarbituric acid reaction and was measured at 532 nm in the UV-Spectrophotometer[18]. The amount of Thiobarbituric Acid Reactive Substance (TBARS) was calculated by using an extinction coefficient of 1.56 x 105/M/cm and expressed as nmol TBARS formed /mg protein.

GLUTATHIONE S-TRANSFERASE (GST)

Activity of Glutathione *S*-transferase (GST) was assayed at 340 nm by measuring the increase in absorbance using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate[19]. The results were expressed as nM of GSH and CDNB conjugate formed /min/mg protein. Values expressed as nanomoles of reduced glutathione and CDNB conjugate formed/min/mg protein.

CATALASE (CAT)

Catalase (CAT) activity was measured at 240 nm by determining the decay of hydrogen peroxide levels and was expressed as µmol of hydrogen peroxide consumed /min/mg/protein[20].

REDUCED GLUTATHIONE (GSH)

The reduced glutathione (GSH) was measured at 412 nm using 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent [21]. The values were expressed as µmol of GSH oxidized/mg protein.

ACETYLCHOLINESTERASE ACTIVITY (AChE)

Acetylcholinesterase activity (AChE) activity was determined using Ellman's reagent, DTNB (5, 5'-dithio-bis (2-nitrobenzoic acid); 0.5mM) and acetylthiocholine iodide (ACTI) as substrate [22, 23, 24]. The rate of change of absorbance at 412 nm was recorded over 1.5 minutes at 25°C.

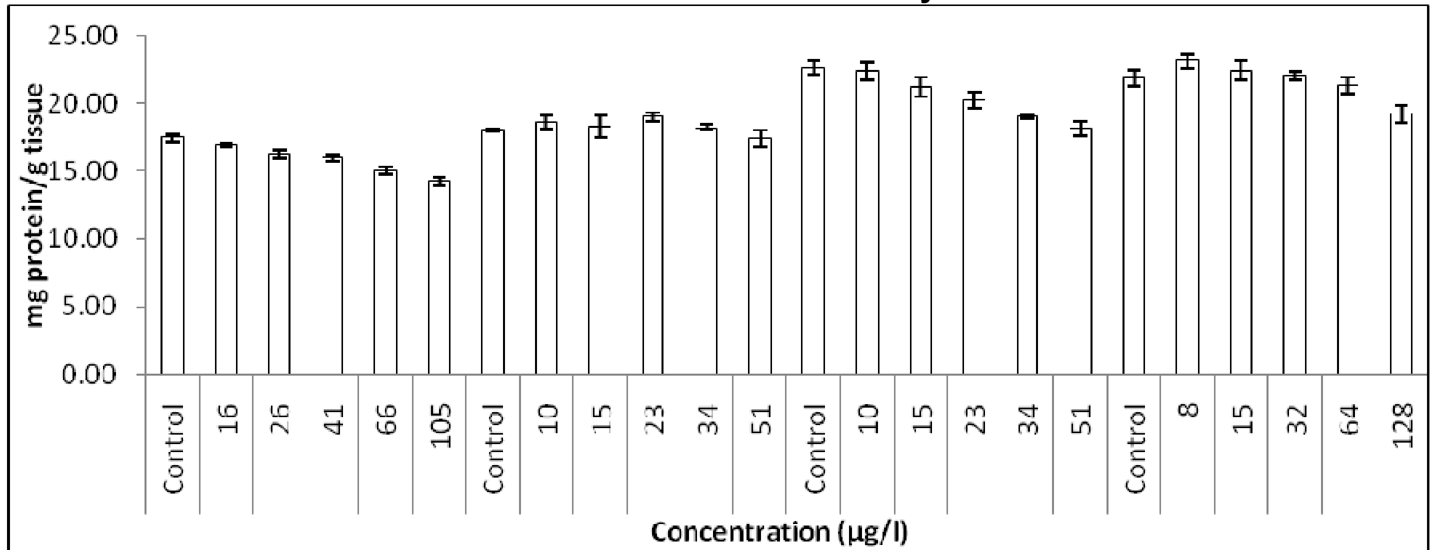
TOTAL PROTEIN

The protein concentration of each of the sample extract was determined measured at 750 nm in UV-Spectrophotometer[25].

RESULTS

The level of total protein in the *P.viridis* significantly (*P*<0.001) decreased along with the concentration (Graph 1).

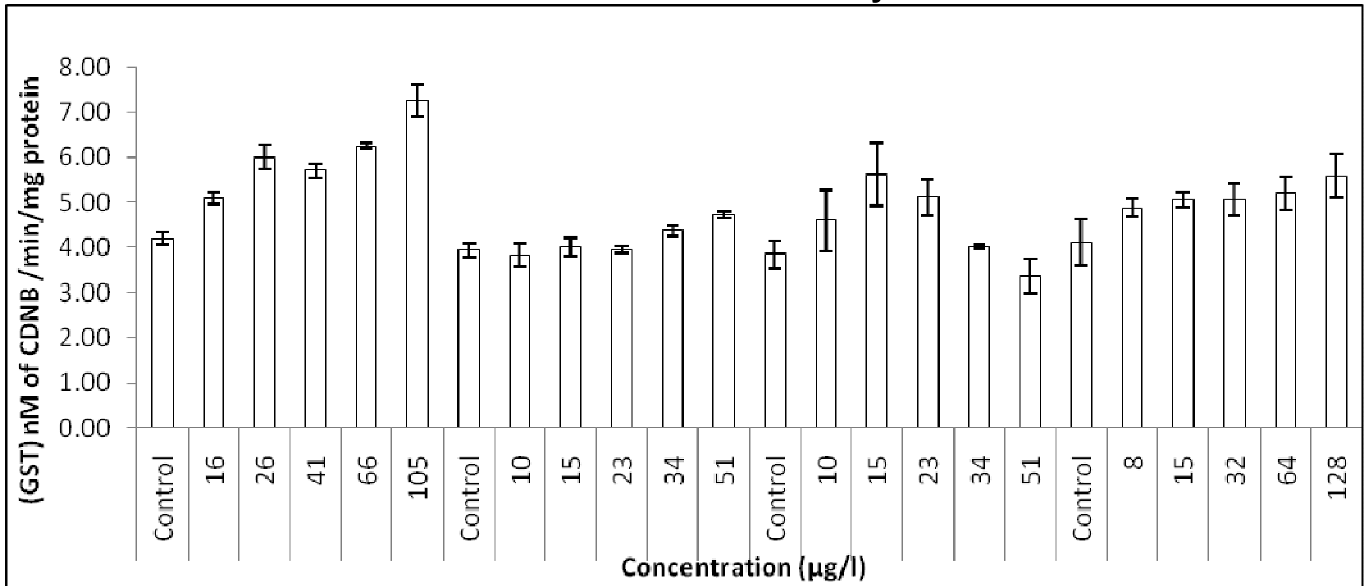
Graph 1
Variation of total level of protein in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of P<0.05, One-way ANOVA.

Significant (*P*<0.001) increase in the activity of GST and CAT (Graph 2 & 4). The level of GSH decreased with no linearity. The LPO level in the tissues of the *P.viridis* showed a significant (*P*<0.001) increase with increase in concentration of cadmium (Graph 6). The activity of AChE significantly (*P*<0.001) decreased. *P.viridis* exposed to copper showed no significant changes in the level of total protein. It was also observed in the activity of GST (Graph 2). Copper concentration of 10, 15 and 23 µg/l produced significant (*P*<0.05) decrease in the level of GSH. AChE level significantly (*P*<0.001) decreased in the activity along with increase in concentration (Graph 5). CAT activity and LPO level in the tissues decreased in concentrations (10, 15, 23 and 34 µg/l) and increased in the 51 µg/l significantly (*P*<0.05) (Graph 4 & 6). *P.viridis* exposed to lead in the short-term chronic toxicity test revealed that the level of protein significantly decreased (Graph 1). The effect in the activity of GST was induced by 15 µg/l significantly (*P*<0.05) (Graph 2).

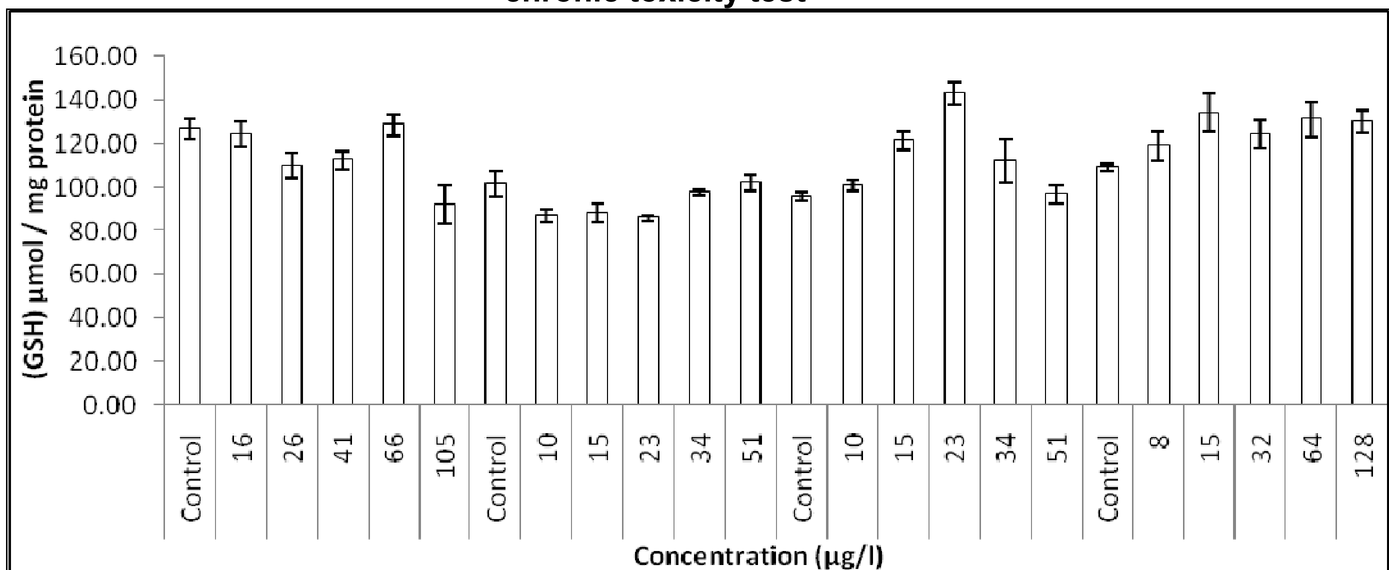
Graph 2
Variation of antioxidant enzyme (GST) in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of P<0.05, One-way ANOVA.

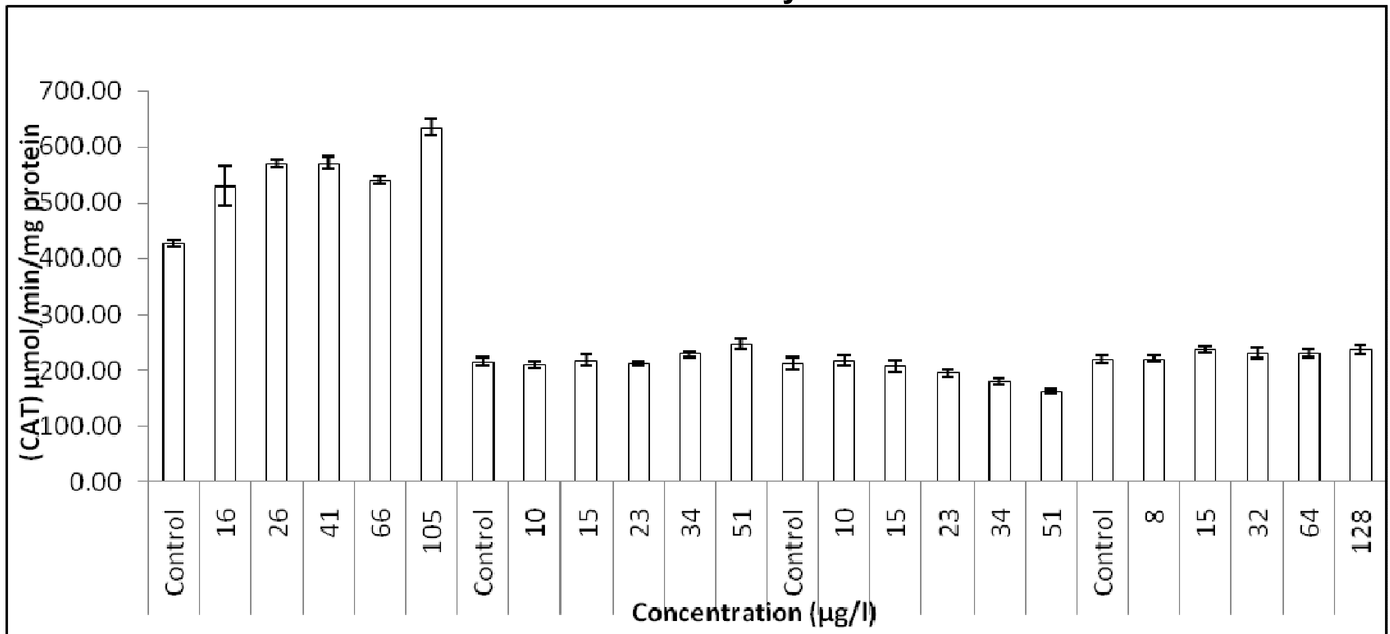
Significantly (*P*<0.001) the level of GSH increased in 23 µg/l, also in 15 µg/l (*P*<0.05) of lead concentration (Graph 3). CAT activity was significantly enhanced in the 34 and 51 µg/l lead concentration (Graph 4). AChE activity and LPO level was highly enhanced by marked increase and decrease in increasing concentration was statistical significant at *P*<0.001 (Graph 5 & 6).

Graph 3
Variation of GSH in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



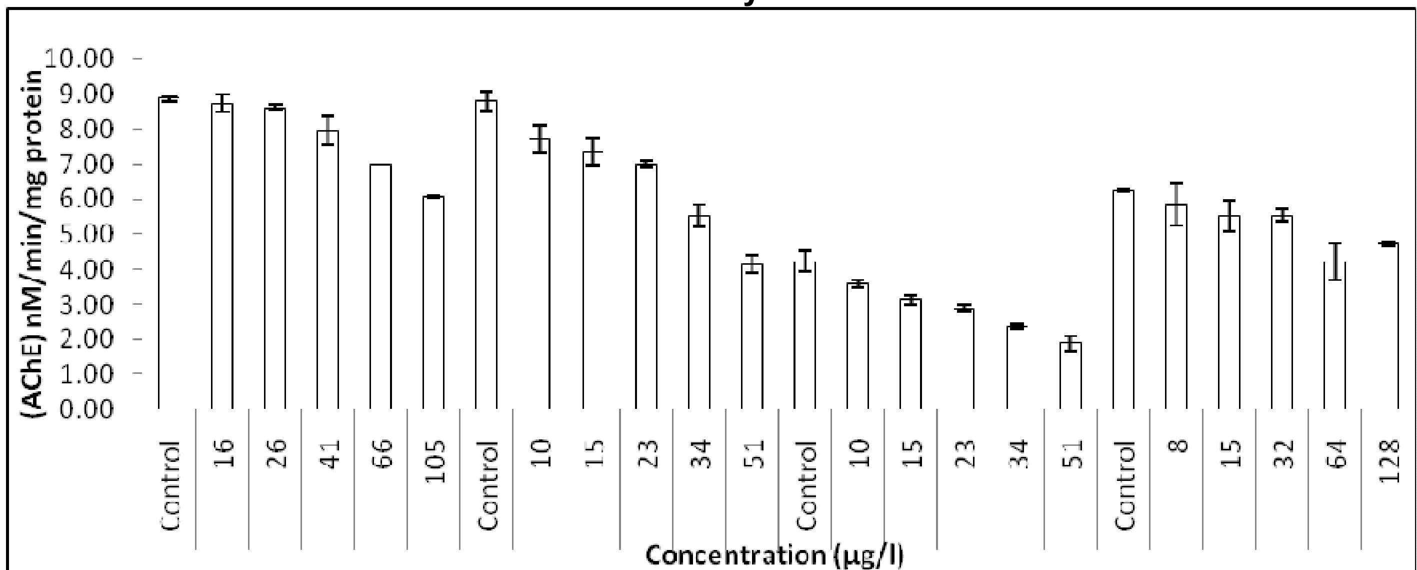
Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of *P*<0.05, One-way ANOVA.

Graph 4
Variation of catalase in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



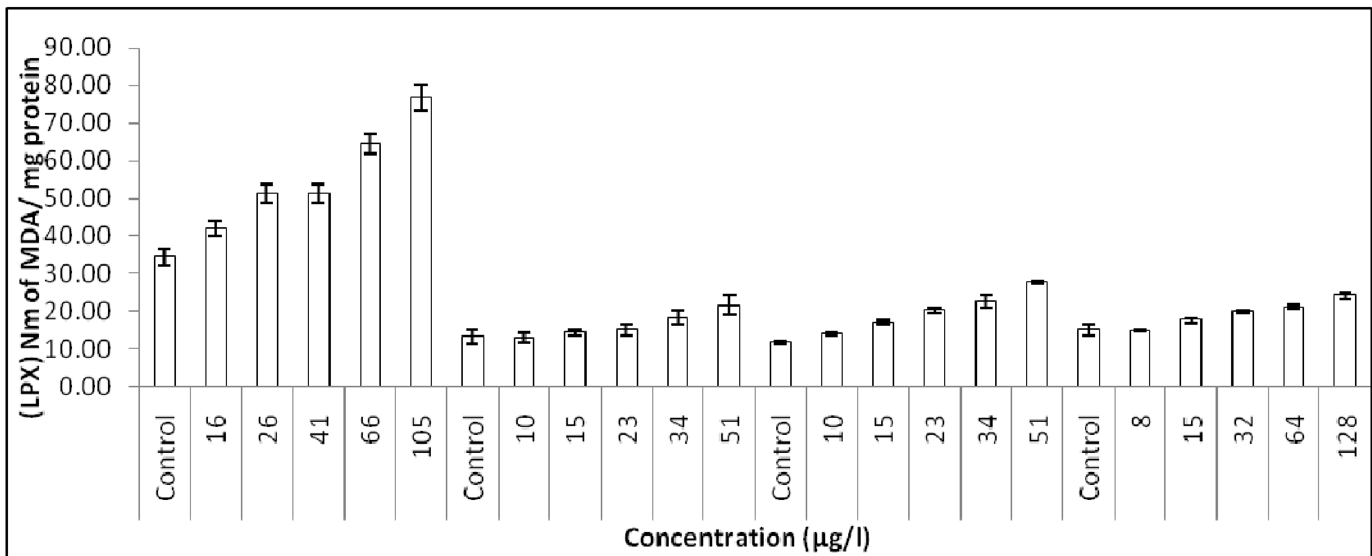
Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of P<0.05, One-way ANOVA.

Graph 5
Variation of AChE in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of P<0.05, One-way ANOVA.

Graph 6
Variation of lipid peroxidation in *P.viridis* exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test



Mean error bars in the graph represents the mean ±standard deviations from duplicate samples, significance of P<0.05, One-way ANOVA.

The total level of protein, GST and CAT activity had variation with no linearity in the zinc treated *P.viridis* (Graph 2 & 4). Significant (*P*<0.05) decrease in the activity of AChE was found in 128 µg/l (Graph 5). The level of GSH in the tissues of *P.viridis* exposed to zinc showed significant (*P*<0.05) increase trend with increase in zinc concentrations (Graph 3). The trend was similar in the level of LPO whose significance (*P*<0.01) was recorded at 128 µg/l (Graph 6).

DISCUSSION

P.viridis exposed in lab to metals showed changes in LPO and antioxidant levels[26]. In bivalves increased CAT activity is associated with tissue burden accompanied by heavy metals[27]. CAT is the primary scavengers of H_2O_2 in the cell. Increased CAT activity presently seen exposed to trace metals in *P.viridis*, further indicate that pollution stress has elevated the formation rate of H_2O_2. These results are comparable to those found in other studies, where CAT activity increases at sites contaminated with metals[28]. Heavy metals could induce increased CAT activity in bivalves[26]. Elevated reduced glutathione (GSH) activity was observed in molluscs exposed to petrochemical products[29]. Increased GST activity exposed to zinc and copper suggests that higher pollutant stress may have induced GST expression in order to protect tissues from xenobiotic damage[19].

Presence of contaminants are likely responsible for elevated GST activity in tissues of *P. viridis*. A depletion of GSH observed in the tissues exposed to lead and cadmium suggest greater utilization of GSH in order to combat metals present in these marine habitats. The metal detoxification process is directed through thiolate sulfur atom present in GSH, thus representing the first line of defense against heavy metal cytotoxicity, which may be responsible for reduced GSH content, observed presently[30].

Lead alters filtration and assimilation rates[31], changes expression of stress proteins[32] and decreases immunity in mussels[33]. In addition, lead binds with glutathione and decreases GSH level[34]. Similar responses of decreased GSH level have been shown in the mussel *Perna perna*, as an indicator of oxidative stress[35]. Lead induces oxidative stress in mussels, suggesting that lead, along with other metals, can induce LPO and production of ROS[36], Low GSH levels were observed in

mussels indicated utilization of antioxidants for detoxification processes[37].

Variations of CAT activity are consistent with other findings, showing decreased and increased activities exposed to cadmium, copper, lead and zinc[38]. Elevated activity of CAT was reported in *Mytilus galloprovincialis* in the Adriatic Sea[39]. Seasonal alterations in the CAT activities were measured in the digestive gland of the brown mussel *P.perna*[40]. The effects of trace metals on antioxidant defence enzymes are more evident when different species are compared. Trace metals seem to influence directly on the antioxidant defence enzyme due to biological factors which regulate fluctuations of defences[41].

The removal of H_2O_2 is an important strategy of marine organisms against oxidative stress[42]. Increased activities of CAT have been reported in several fish and invertebrate species[43]. CAT activity in mussels is not sufficient to eliminate H_2O_2 before the formation of hydroxyl radicals as it has been suggested by others[41]. Increased concentration of LPO was observed in mussels exposed in polluted areas when compared to less polluted sites[44]. Concentration of LPO was significantly higher ($P< 0.05$) in higher concentrations of cadmium, copper, lead and zinc. CAT activity was reduced due to increased levels of exposure indicating the importance of antioxidant[44].

CONCLUSION

Alterations in antioxidant enzyme activities and other biomarkers of oxidative stress in *Perna viridis* may cause biochemical dysfunction. In addition, the results provide evidence that enzyme and non enzyme biomarkers of oxidative stress can be sensitive indicators of aquatic pollution caused by heavy metals. Our results indicate a significant elevation in lipid peroxidation, glutathione *S*-transferase, catalase, reduced glutathione (GSH), acetylcholinesterase and total proteins in *P.viridis*. The apparent increase in lipid peroxidation may be attributed to the accumulation of the heavy metals in the tissues of *P.viridis*. Metal catalyzed formation of ROS capable of damaging proteins and lipids was well documented.

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