

Response of DNA, Proteins, Lipids and Antioxidant Enzymes as Measure of Toxicity to Mercury Exposures in Green Mussel *Perna viridis*

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ABSTRACT

Studies on exposures of gills of green-lipped mussel *Perna viridis* to sublethal levels of mercury (Hg) indicates that oxidative stress marker like lipid peroxidation and protein carbonyl content increase. With the exception of superoxide dismutase, the activities of catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase also showed higher values. Reduced glutathione and H₂O₂ level gradually increased throughout the experimental period. Ascorbic acid content of the gill tissue was positively correlated with SOD activity. Similarly, a positive correlation was shown by lipid peroxidation and H₂O₂ levels. The DNA strand breaks observed on the 5th day of the Hg exposure, regained later. These properties of gill tissue could effectively be utilized to measure sub lethal toxicity of Hg pollution during environmental monitoring studies.

Key words : Green-lipped mussel ; *Perna viridis*; Oxidative stress; antioxidant enzymes; reduced glutathione; DNA strand break.

INTRODUCTION

Marine communities in the coastal waters along Indian coast are often stressed by discharges of variety of chemicals and organic materials derived from industries and metropolitan cities (Karunasagara *et al.*, 2005 ; Jha *et al.* , 2003 ; Shailaja and D'Silva, 2003. Among the chemical substances reaching these waters, trace metals play a vital role in determining the health of the fishery resources. Accumulation of trace metals in waters and subsurface sediments are often recorded along the Coromandal coast, Pondichery, Chennai and Vishakhapatana along the east and Cochi, Mumbai and Gujarat along the west coast of India (Zingede, 2001; Ramaiah *et al.*, 1998;

Gajbhiye *et al.*, 1995 ; Varshney and Govindan, 1995; Panigrahy *et al.*, 1999). The waters around Mumbai city, where about 2,200 MLD domestic wastes are pumped out, retained high mercury content varying from 0.12 to 1.4 µg/l in water, 0.08 to 0.36 µg/g dry wt in sediments and 0.217 to 0.512 µg/g wet wt in fish muscle (Sanjiri *et al.*, 1988). Similarly off Karwar where polluting industries are located, mercury was recorded as high as 2.68 µg/l in water and 1.32 µg/g dry wt in sediments. Such examples are not uncommon in the other Asian and European countries. Shi *et al.*, (2005) reports that mercury concentrations in Dagu river sediments in China, reach as high as 8779.1 µg /g (dry weight) as a result of effluents of Tianjin city with a population of 9.16 million with daily industrial

and domestic discharges of 4.42×10^9 and $3.57 \times 10^9 \text{ m}^3$. Similarly, Medina et al. (1986) have shown accumulation of Hg in water column in Celtic Sea, on the western European continental margin. While Ando et al., (2002) has reported Hg accumulation to $238 \mu\text{g/g}$ in body muscle of vestimentiferan worm, *Lamellibrachia satsuma*, near hydrothermal vents at a depth of 80–100 m in the northern parts of Kagoshima Bay.

Stresses arising out of metal contamination could be varying including accumulation in vital organs and modulation of their cellular enzymatic function (Livingstone et al., 1990; Adema et al., 1991, Cheung et al., 2002). Moreover self purification processes in the form of detoxification mechanisms, also play an equally important role in reducing the metal toxicity (Dunn, 1987; Romero-Isart and Visak, 2002). These properties of marine organisms could be used as biomarkers to detect metal contamination at sub-lethal levels for the control of pollution and save the communities from total destruction.

The exposure of marine mollusk to metals has been shown to induce oxidative stress through the formation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipid-peroxidation (Viarengo et al., 1990; Dafre et al., 2004). An imbalance among these reactions can lead to a condition called oxidative stress (Halliwell and Gutteridge, 1986). ROS such as superoxide radical (O_2^-), hydroxyl radical ($\cdot\text{OH}$), H_2O_2 and other free radicals derived from oxygen attack almost all bio-molecules present in their vicinity including the phospholipids, proteins and DNA, which result in lipid peroxidation, protein oxidation and DNA damage (Halliwell and Gutteridge, 1986; Labieniec and Gabrylak, 2004).

Among trace metals mercury is considered as the most toxic pollutant having bioaccumulation capabilities in marine animals such as bivalve mussel (Kureishy and D'Silva, 1993). Several studies are carried out on the accumulation of mercury in mussel tissue (Lakshman and Nambisan, 1979; Krishnakumar et al., 1987). But not much information exists on the influence of mercury on anti-oxidative defense system in gills of *P. viridis* and its effects on bio-molecules. In these studies, attention has been given to variation in the antioxidant defence system and DNA damage, as measure of Hg toxicity to the green mussel *Perna viridis*.

MATERIAL AND METHODS

Experimental animals and treatment

Green-lipped mussels (*Perna viridis*), 8-10 cm were collected from Bambolim beach Goa, India relatively far less polluted place and transported to the laboratory in polythene bags filled with natural habitat sea water. Mussels were acclimatized in aerated sea water for 1-2 week. and were exposed to $1/10$ of LC_{50} of mercury chloride ($\text{LC}_{50} = 0.450 \text{ mg / lit}$) up to 15 days. The control group was maintained under similar experimental conditions. The animals were sacrificed after 5th, 10th and 15th day of treatment. The sea water containing metals (1.5 L / animal) was changed daily.

Sample preparations

The digestive gland was carefully excised, surface dried with filter paper, thoroughly washed with phosphate buffer pH 7.4 and homogenized with 50 mM phosphate buffer pH 7.4 containing, 1 mM EDTA, 1 mM DTT, 0.15M KCl, 0.01% PMSF. Homogenization was carried out at 4°C using 12-15 strokes of a motor driven Teflon Potter

homogenizer and centrifuged at 10000 rpm. The post mitochondrial fraction was taken for biochemical studies (SOD, CAT, GST, GPx, GR assay, lipid peroxidation and hydrogen peroxide).

Estimation of lipid peroxidation

The tissue samples were assayed according to the method of Ohkawa *et al.*, (1979) with minor modifications. In brief, the reaction mixture containing approximately 1 mg of protein, 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA), 1.5 ml of 20% acetic acid adjusted to pH 3.5 with NaOH, 0.2 ml of (8.1%) sodium dodecyl sulfate (SDS), 0.2 ml of double distilled water and 0.1 ml of (0.76%) butylated hydroxytoluene (BHT) was heated at 95°C for 60 min, then cooled to room temperature and centrifuged at 2,000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm. The amount of TBARS formed was expressed as nmol TBARS formed per mg protein.

Estimation of protein carbonyl

Protein oxidation was detected by quantitating the cellular protein carbonyl content, according to Mecocci *et al.*, (1999). Briefly 800 ml of 10 mM DNPH was added to 800 ml of sample containing a known concentration of protein (approximately 2 mg protein). Sample blanks were prepared using 800 ml of sample with 800 ml of 2.5 M HCl with no DNPH. The tubes were kept in dark and vortexed every 15 min for 1hr. Equal volumes (1.6 ml) of 20% TCA was added to each tube and was kept 10 min at room temperature and after centrifuge, the pellets are washed three times with 1 ml of ethanol-ethylacetate mixture (1:1). The protein was solubilized in 2 ml of 6M guanidine hydrochloride. The carbonyl content was

calculated from the absorbance measurement at 366 nm with the use of molar absorption coefficient of 22,000 mol.cm⁻¹ and expressed as nmol/ mg protein.

Alkaline DNA unwinding assay

Alkaline DNA unwinding assay was determined according to Shugart (1988 a, 1988 b). The gill tissue was homogenized in 1N NH₄OH in 0.2% Triton X-100. The homogenate was transferred to a centrifuge tube containing 2 ml water, 6 ml of Chloroform / Phenol / Isoamylalcohol (24/25/1 v/v) and was allowed to stand for 10-15 min. The homogenate was then centrifuged at 1000 rpm for 20 min at 4°C. The aqueous layer was transferred to a new microcentrifuge tube. The DNA was recovered by Sephadex-G-50 column (1 cm i.d: 4 ml settled bed volume), equilibrated in G-50 buffer (0.15M NaCl, 0.01 M Tris pH 7.4, 1 mM MgCl₂, 5 mM EDTA). About 100 ml of DNA sample was combined with 50µ of 0.05N NaOH and maintained at 4°, 80° and 38°C for 25 min, for fluorescence determination of double stranded DNA (dsDNA), single stranded DNA (ssDNA) and partially unwound DNA (au DNA). After incubation sample was neutralized by addition of 50 ml of 0.05N HCl. This was followed immediately by the addition of 5 µl of 0.2% SDS in 2mM EDTA and mixed properly. The fluorescence of ds DNA, ssDNA, auDNA was measured after addition of 0.2M potassium phosphate buffer (pH 6.9) and Hoechst dye 33258, using a spectrofluorometer (Shimadzu RF-501) [Ex: 360 nm, Em:450 nm].

Estimation of antioxidant enzymes

Superoxide dismutase activity was measured according to the method of Paoletti *et al.*, (1990). Catalase was determined according to the method of Aebi (1974) by the

depletion rate of H_2O_2 at 240 nm in a reaction buffer containing 0.05 M of phosphate buffer pH 7.4 with 12 mM H_2O_2 . Glutathione peroxidase and glutathione reductase activities were measured according to the method of Paglia and Valentine, (1967) and Massey and Williams (1965), respectively. Enzyme activities were expressed as nmol NADPH oxidized / min/mg protein. Total GPx activity was measured taking cumine-hydroperoxide as the substrate, whereas the Se-dependent enzyme activity was assayed taking t-butylhydroperoxide as substrate. The enzyme GST was measured at 340 nm according to Habig *et al.* (1974) using 0.3 mM CDNB as substrate.

Estimation of small antioxidant molecules

A 20% tissue homogenate was prepared by 5% trichloro-acetic acid and was centrifuged 2000 rpm for 10 min. the deproteinised supernatant was used for assay of GSH using 0.6 mM DTNB in 0.1M Phosphate buffer pH 8.0 and the formation of thiolanion was measured at 412 nm Ellman, 1959, Ascorbic acid (ASA) was measured the stoichiometric reduction of phosphomolybdate by ASA Mitusi and Ohata, 1961(Rewrite).

Estimation of hydrogen peroxide

The hydrogen peroxide content in postmitochondrial fractions was determined spectrophotometrically according to the methods of Pick and Keisari (1981) using horseradish peroxidase and phenol red. Protein content was estimated by the Folin-Phenol reaction as described by Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical analysis

Results were expressed as mean \pm

standard deviation (SD). Difference among the means of control and treatment was analyzed by Student's t-test. Correlation analyses were also conducted to find the relation between various parameters. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The level of lipid peroxidation in gills measured as TBARS formed due to mercury exposure showed higher values than control till the end of the experiment (**Fig-1A**). Significant increase in carbonyl content on 10th day (**Fig-1B**), suggests that mercury becomes toxic and cause oxidation of proteins. With the exception of SOD activities (**Fig-1F**), the other antioxidant enzymes such as CAT, GST, GR and GPx showed fluctuations with increasing values (**Fig-2D**) above control at certain occasions (**Fig-1E, 2A, 2B & 2C**). However increase in GSH content and progressive decrease in ASA level progressively decreased (**Fig-2E**) was seen from 5th to 15th day in gills. Elevated levels of H_2O_2 were also observed in Hg treated gills (**Fig-1D**). But the level of DNA double strands measured as DNA unwinding assay, substantially reduced on 5th day in Hg exposed specimen which increased later (**Fig-1C**).

DISCUSSION

Attack by molecular oxygen produces lipid peroxyradical that can obstruct a hydrogen atom from an adjacent lipid to form a lipid hydroperoxides (Halliwell and Gutteridge, 1986). This process affects membrane fluidity and integrity of bio-molecules associated with membrane can be quantified by measuring TBARS assay. Elevated levels of TBARS in the present study in gills of mercury exposed of mussels (Fig-1A), was similar to those reported

earlier for mollusks, human erythrocyte and rats (Géret *et al.* 2002; Kee and Sin, 1992; Bansal *et al.* 1992). TBARS induction values showed good correlation with H_2O_2 contents ($r = 688$, $p < 0.05$; Fig-3A), which generate extremely reactive molecule such as OH (Storey, 1996), Cavaletto *et al.* (2002) reported that the pro-oxidant H_2O_2 increases lipid peroxidation and lysosomes / cytoplasm size ratio (which marked alteration of the physiology of the lysosomal vacuolar system). H_2O_2 stimulates intra-cellular signal cascade, such as mitogen- activated protein kinase and activities of transcription factors (Nose, 1991). Increased levels of H_2O_2 as compared to control seen in present study can therefore be considered as the marker of metal pollution.

During normal physiological conditions, antioxidant enzymes get activated to protect cells and tissues from oxidative injury. Superoxide dismutase scavenges superoxide radical ($O_2^{\cdot-}$) to H_2O_2 , which further gets neutralized by catalase or glutathione peroxidase. Exposure to contaminants generate ROS which may induce or inhibit the antioxidant defense mechanism of the animals. The significant inhibition of SOD activity in mercury exposed mussel in the present study could reflect the inability of cells to eliminate $O_2^{\cdot-}$. This means that redox defense system in the gill gets inactivated after mercury exposure. The gill is expected to be more sensitive to oxidative injury compared to other organs, since it serves as the front line of contact with potential contaminants or pollutants. Doyatte *et al.* (1997) on exposure of mussel *U.temidis* to copper reported that SOD remains unchanged. Similar results were also obtained by Torres *et al.* (2002) *i.e.*, with the exception of SOD activity, the other antioxidant enzymes showed

significantly higher levels at the polluted sites. These observations suggest that SOD could remain inactive with certain pollutants. Furthermore when SOD values were plotted vs ascorbic acid (ASA) content it showed a positive correlation ($r = +0.581$, $p < 0.05$, Fig-3C) suggesting that ASA might play an important role in protecting the organisms from ROS during mercury exposure of gill tissue. According to Halliwell and Gutteridge (1986) ASA can also scavenge $O_2^{\cdot-}$ in the cellular system. This suggests that ASA could have compensated the function of SOD in the present study. In addition to the antioxidant functions, ASA also acts as crucial micronutrients and perform various important physiological functions like collagen synthesis and formation of connective tissue (Wilson and Poe, 1973). Moreover the increased values shown by other antioxidant enzymes such as CAT, GPX, GR and GST in the present investigations could help in controlling the oxidative damage caused by mercury. The importance of CAT as an antioxidant lies in its involvement with the Haber-Weiss reaction, which removes H_2O_2 to generate extremely reactive molecule such as OH (Storey, 1996). The increased CAT activity (Fig-1E), on the 15th day of exposure might indicate that cells were subjected to ROS. The elevated glutathione reductase activity on 5th to 15th day can be related to the thiol status of cells which leads to regenerate GSH from GSSG, resulting in increased level of GSH observed during experimental period. Reduced glutathione (GSH) is one of the main soluble antioxidants acting both as direct scavengers of oxyradical and as a cofactor for several antioxidant enzymes. De Giulio *et al.* (1995) observed that some metals can bind to GSH and thereby limit its availability to counteract lipid peroxidation and DNA oxidation. Glutathione-S-transferase,

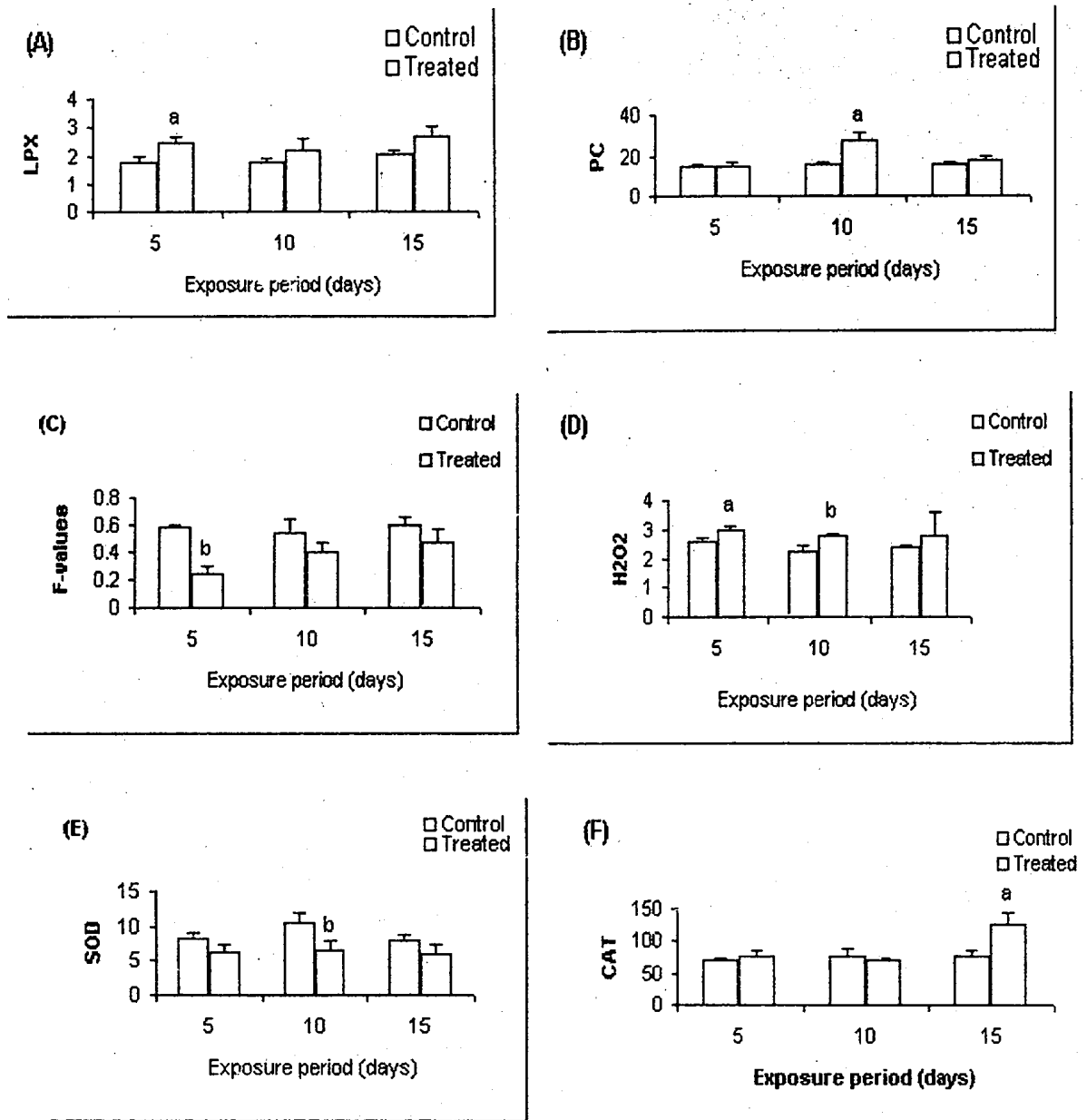


Figure 1: Effect of mercury chloride on (A) lipid peroxidation (nmol TBARS mg protein⁻¹), (B) protein carbonyl (nmol mg protein⁻¹), (C) DNA unwinding assay (F-value), (D) hydrogen peroxide (nmol H₂O₂ mg protein⁻¹), (E) superoxide dismutase (Unit mg protein⁻¹), (F) catalase (nkat mg protein⁻¹) in gills of *P. viridis*. Data are expressed as mean \pm SD (n=3). Denote indicates significant difference between control and treated mussels ^a*P* < 0.05, ^b*P* < 0.01 and ^c*P* < 0.001.

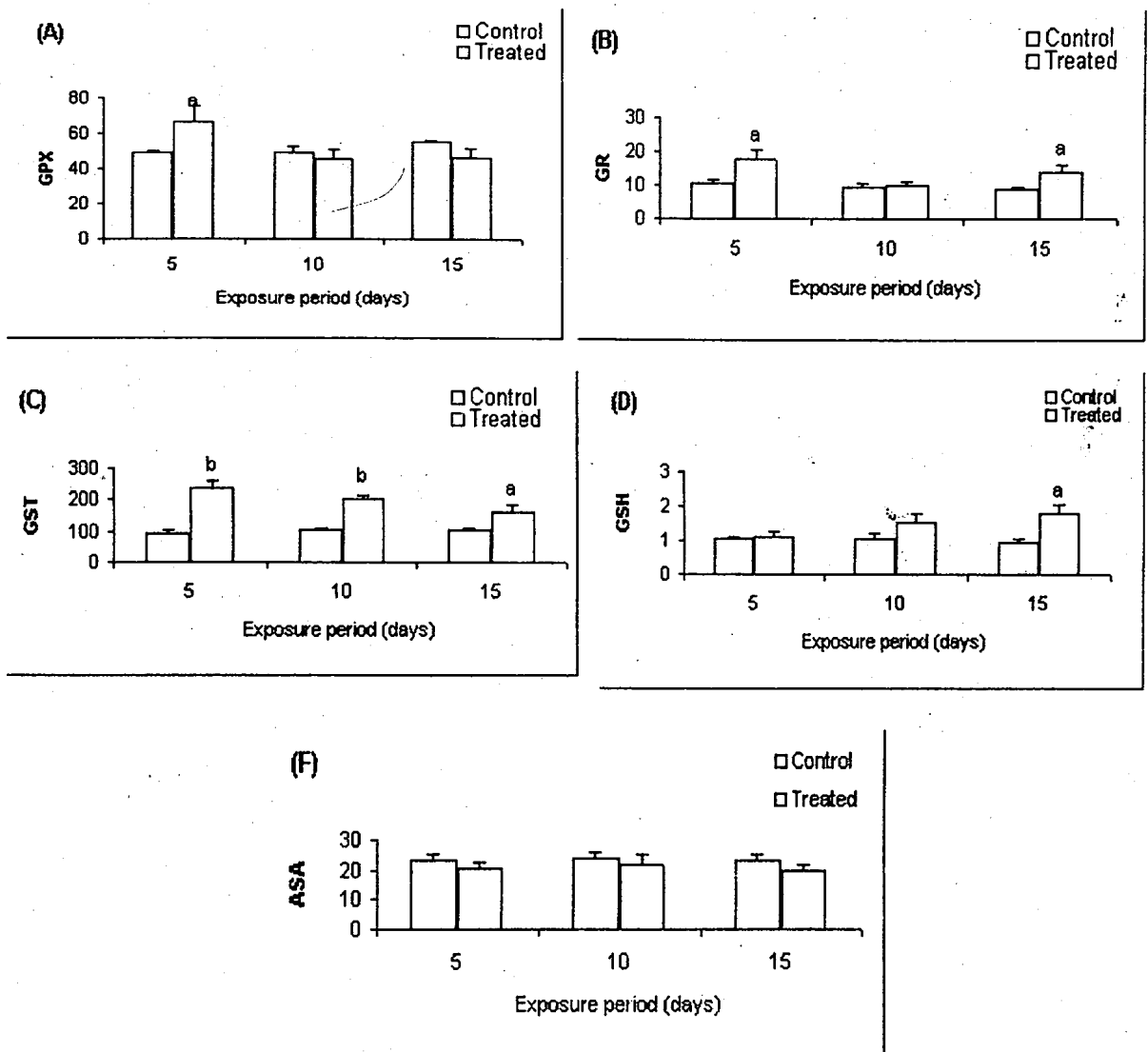


Figure 2: Effect of mercury chloride on (A) glutathione peroxidase (nmol NADPH oxidized min⁻¹ mg protein⁻¹), (B) glutathione reductase (nmol NADPH oxidized min⁻¹ mg protein⁻¹), (C) glutathione-S-transferase (nmol CDNB conjugate formed min⁻¹ mg protein⁻¹), (D) reduced glutathione (μmol GSH gm wt. tissue⁻¹) and (E) ascorbic acid (μg ASA gm wt. tissue⁻¹) in gills of *P. viridis*. Data are expressed as mean ± SD (n=3). Denote indicates significant difference between control and treated mussels ^a*P* < 0.05, ^b*P* < 0.01 and ^c*P* < 0.001.

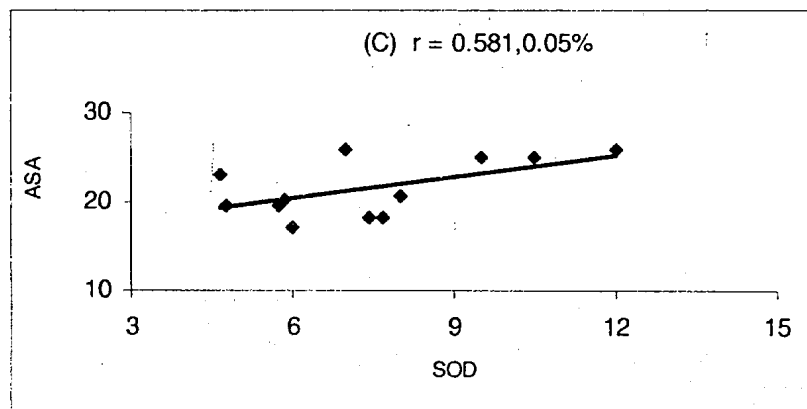
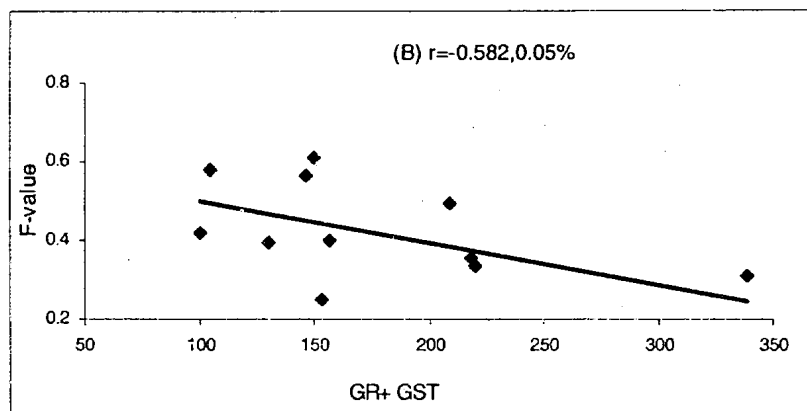
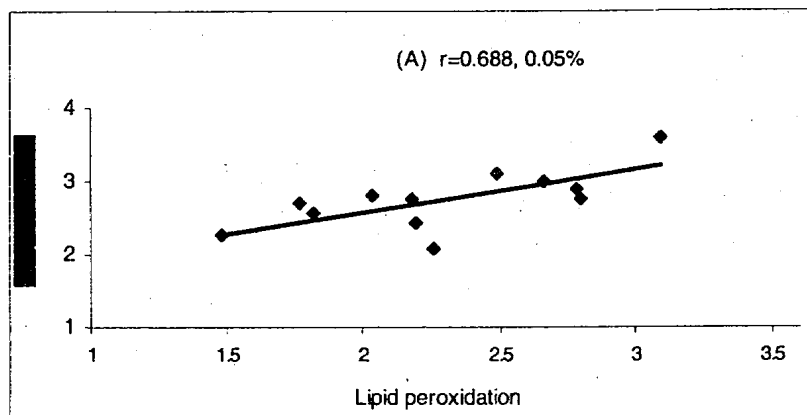


Fig-3. (A) Correlation curves between Lpx (nmol/ mg protein) level vs hydrogen peroxide (nmol/ mg protein) (B) between F-value vs GR (nmol NADPH oxidized / min /mg protein) +GST (nmol CDNB conjugated formed/ mg protein) (C) between ASA (ig / gm wt. tissue) vs SOD (U / mg protein).

phase II detoxifying enzyme systems has been used as a biomarker of organic industrial pollutants (Fitzpatrick *et al.* 1997). Increase in activity as seen in present study was also reported by Torres *et al.* (2002) during field observations in *Mytella guyanensis* from polluted mangroves, Brazil. Higher level of GSH, GST and GR activities in *P. viridis* during the experimental period suggest that these mollusks more effectively use glutathione metabolism path way as an adaptive response against ROS generated by mercury.

Protein oxidation products and carbonyl derivatives result from oxidative modification of amino acid, side chains and reactive oxygen-mediated peptide cleavage which react with lipid and carbohydrates (Labieniec and Gabrylak, 2004). Generation of protein carbonyl compounds during experimental period, as seen in Fig-1B may indicate that proteins subjected to free radical damage. De Zwart *et al.* (1998) reported that the protein carbonyl content of cells and tissues is associated with a number of pathological disorders. These results are consistent with our earlier observations that exposure of mercury to hepatic mitochondria of *P. viridis* showing protein aggregation and other changes such as mitochondrial swelling and lipid peroxidation (Data not shown).

Like lipid membranes and proteins, DNA is other target macromolecules in cellular oxidative injury. The reaction between DNA macromolecules and free radical species leads to damaged base pairs, strand cross-links and strand breaks. The level of strand breaks in cellular DNA has been proposed as a biomarker in environmental bio-monitoring (Shugart, 1990).

Several field and laboratory studies also showed the damaging effects of pollutants on DNA molecules (Siu *et al.*, 2003; Black *et al.*, 1996; Labieniec and Gabrylak 2004). A treatment with (45 $\mu\text{g/L}$.) mercury induces DNA strand break during initial experimental periods (Fig-1C). This would lead to very high level of DNA damage due to oxidative stress, but the repair mechanism appears to have set in increasing DNA integrity from 10th to 15th day. This is further confirmed by the positive correlation between F-values (Fractions of ds DNA) and antioxidant enzymes (Fig-3B). The level of DNA damage is depending upon the DNA repair mechanism of cells, also depend on uptake, distributions, storage, bio-transformation and elimination of toxicants (Black *et al.*, 1996) It appears therefore that antioxidants could have reduced the toxicity to the cell DNA by bio-transformation process. Thus, the changes of these antioxidant enzymes and the DNA damage could serve as a measure of toxicity of the sublethal exposures to Hg pollution.

CONCLUSION

The mussel exposure to mercury showed marked difference in both enzymatic and non-enzymatic antioxidant response in *P. viridis*. Differences in the protective behavior of mollusk were also evident by the ROS effects such as decreased level of protein carbonyl and DNA strand break. Higher content of GSH and antioxidant enzymes serves as protective mechanism for tissues from mercury induced toxicity. The results clearly indicate that these variations can be used as markers of pollution in environmental monitoring studies.

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