

Author version: Chemosphere, vol.71(10); 1977-1985p

**Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.),  
on mercury exposures**

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## **Abstract**

Sub-lethal effects of mercury exposure ( $1/10$  th of  $LC_{50}$  i.e  $0.045 \text{ mg l}^{-1}$ ) for 5, 10 and 15 d was investigated on oxidative stress parameters and antioxidant defences in digestive gland of *Perna viridis*. In addition to this an *in vitro* effect of mercury single and supplementation of reduced glutathione on lipid peroxidation was studied. Increased lipid peroxidation (during first 10 days and also during *in vitro* exposures), protein carbonyl and hydrogen peroxides (from 5<sup>th</sup> till last day of exposure) indicates the resultant oxidative stress in the mercury exposed specimen. DNA damage response although less distinct on 5<sup>th</sup> and 15<sup>th</sup> d, its low values on 10<sup>th</sup> d and significant correlation with hydrogen peroxide suggests the toxic role of free radicals towards DNA integrity. Superoxide dismutase, which remains low initially (5<sup>th</sup> d) and increases later suggests its immediate response against oxyradicals. Higher activities of catalase, glutathione peroxidase and glutathione reductase on 15<sup>th</sup> d and glutathione-S-transferase from 10<sup>th</sup> d onwards suggests the adaptive behaviour of the tissue against oxyradicals. Increasing levels of non-enzymatic antioxidant molecules, such as reduced glutathione and ascorbic acid indicated its involvement in counteracting oxidative damage. Further role of reduced glutathione in reducing Hg toxicity is evident in *in vitro* experiments where lipid peroxidation remains low in mercury concentrations supplemented with reduced glutathione. The elevated levels of metallothionein from 5<sup>th</sup> to 10<sup>th</sup> d suggest involvement of this protein in detoxification of reactive oxygen species and toxic metal. The above results suggest that both enzymatic and non-enzymatic antioxidants play an important role in protecting cell against Hg toxicity, which can be used as a biomarker of metal contamination in aquatic environment.

**Key words:** *Perna viridis*; mercury; antioxidant defences system.

## 1. Introduction

Estuarine and coastal waters are often at the risk of pollution of toxic metals such as mercury from natural as well as anthropogenic sources (Nanda, 1993; EPA, 1997; Das et al., 2001). In India Chloroalkali plants are the main source for mercury release to the atmosphere and surface waters. Besides, this coal-fired plants such as thermal power, steel, and cement plants also contribute significantly to mercury pollution (Nanda, 1993). Contaminated sediments in Kodaikkanal, in India, Minamata Bay and Fukuro Bay in Japan and Dagu River in China have concentrated Hg up to 0.350, 4.3, 4.8 and 8.8 mg kg<sup>-1</sup>, respectively (Jian-bo et al., 2005; Karunasagar et al., 2006; Tomiyasu et al., 2006). Accumulation of Hg in water has been reported to be up to 103.5 µg l<sup>-1</sup> in the Rushikulya River Estuary in India (Shaw et al., 1986). In Ennore Estuary, Chennai, Tamil Nadu the distribution pattern of mercury was 0.0763 µg/l in water, 0.0428 µg g<sup>-1</sup> in sediment and 0.013 to 0.40 µg g<sup>-1</sup> wet wt in fish due to the discharge of industrial effluents. (Rajathy, 1997). Along the west coast of India the mercury in seawater was up to 0.116 off Cape Comorin and Laccadive Sea in the south and 0.352 µg l<sup>-1</sup> off Veraval coast in the North (Kaladharan et. al., 1999).

It has been reported that *P. viridis* exposed to 0.2 mg l<sup>-1</sup> could accumulate around 21.63 µg g<sup>-1</sup> Hg in tissue within 4 d of exposure (Lakshmanan and Nambisan 1989). Mercury which is a non-essential metal, can exist in various oxidation states that are highly soluble, reactive (Heaton et al., 2005), and toxic to bivalves at low concentrations. Cell damage induced by heavy metals is commonly associated with the ROS formation (Leonard et al., 2004), which needs to counteract by defense systems. Antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST), non enzymatic antioxidant molecules such as reduced glutathione (GSH) and metallothionein (MT) can be influenced by external stimuli (Halliwell and Gutteridge, 2001; Manduzio et al., 2005). Hence these parameters could serve as indices to study impact of toxic chemical exposures on organisms.

Toxic effects of Hg to bivalve mollusks studied so far include imbalance of Ca<sup>2+</sup> signaling pathway (Burlando et al., 2004), immunotoxicity (Gagnaire et al., 2004), genetic abnormalities (Yap et al., 2004), reduction in filtration rate, alteration in gill structure (Gregory et al., 2002) and inhibition of Ca<sup>2+</sup>-ATPase activity (Pattnaik et al., 2007). However these studies pertain to specific responses to

understand the physiological behaviour of the animals and not restricted to a single species. In bivalves the antioxidant responses are influenced by both abiotic and biotic factors and may vary in different species (Filho et al., 2001; Lau et al., 2004; Verlecar et al., 2007). This complicates the proper use of antioxidant response as environmental monitoring parameter. Interpretation of field-based effects could well be achieved by laboratory exposures of animals to contaminants. The present studies were therefore initiated to determine an array of responses of Hg toxicity to a single bivalve species *Perna viridis*, which could provide information on the biomolecular damage (lipid, protein and nucleic acid) and antioxidant defence responses to oxidative stress in organisms, which could be used as biomarkers of environmental contamination.

Oxidative stress indices measured at sublethal Hg exposures include lipid peroxidation (LPX), protein carbonyl (PC), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), GSH, ascorbic acid (ASA) and antioxidant enzymes such as SOD, CAT, GPX, GR and GST. In addition DNA strand breaks, as an index of genotoxic stress and MT as adaptive and detoxifying measure were determined in the organisms.

## **2. Material and Methods**

### *Chemicals*

Thiobarbituric acid (TBA), bovine serum albumin (BSA), GR, cumene-hydroperoxide, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), horseradish peroxidase (HRP) and streptomycin sulfate were purchased from Sigma Chemical Co. USA. NADPH, GSSG, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), Phenylmethylsulphonyl fluoride (PMSF) and 2,4-dinitrophenyl hydrazine (DNPH) were obtained from SISCO Research laboratory, India. All other chemicals were analar grade.

### *Experimental animals and their maintenance*

Green-lipped mussels (*Perna viridis*), length 8-10 cm were collected from unpolluted area of Bambolim beach Goa, India. This area is relatively far less polluted because of the absence of any industrial or major fishing activity around it. Mussels were transported to the laboratory in the polythene bags filled with natural habitat seawater. Fouling organisms were removed from the external part of the shells. In the laboratory mussels were acclimatized for two-three weeks in seawater of salinity 30‰, pH 8.0 and temperature range from 23-25 °C. The light and dark regime

was kept constant at 12 h: 12 h. Mussels were fed with unicellular algal species (*Chlorella* sp.) twice daily at a constant initial density ( $2 \times 10^6$  cells / l) during acclimation and exposure periods. To avoid faecal contamination, the water was exchanged every 24 h and oxygen provided by continuous air-bubbling system.

#### *Test solutions and treatment*

Mercury chloride stock solution was prepared in distilled water and desired test solution used in the present study was prepared by diluting the stock solution with filtered seawater.

Under laboratory condition, mussels were kept in fiberglass tank capacity of 120 L. The animals were divided in two groups. Group I animals were exposed to sea water only and served as control for the Group II animals which were exposed to sub-lethal  $\text{HgCl}_2$  ( $1/10$  of  $\text{LC}_{50}$ ) concentration. Sub-lethal Hg levels were determined by obtaining 96 h  $\text{LC}_{50}$  values for  $\text{HgCl}_2$ , which was around  $0.45 \text{ mg l}^{-1}$  for the present species (data not shown). Accordingly sub-lethal concentrations were calculated as  $0.045 \text{ mg l}^{-1}$  ( $1/10$  of  $\text{LC}_{50}$ ). These sub-lethal Hg concentrations were taken in triplicate with 60 mussels in each of the test tanks and the exposure period continued up to 15 d. Seawater in control as well as in the test tanks was exchanged every 24 h, and supplied with the corresponding concentration of mercury. All other environmental conditions were maintained same to those used during acclimation. Animals were sacrificed for biochemical tests after a period of 5, 10 and 15 d of treatment.

#### *Sample preparations*

The digestive gland was carefully excised, surface dried with tissues paper, thoroughly washed with phosphate buffer (50 mM; pH 7.4) and homogenized with 50 mM phosphate buffer pH 7.4 containing, 1mM EDTA, 1 mM Dithiothreitol (DTT), 0.15 M KCl and 0.01% (w / v) PMSF. Homogenization was carried out at 4 °C using 12-15 strokes of a motor driven Teflon Potter homogenizer and centrifuged at 10000g for 20 min at 4 °C. The supernatant was used for biochemical analysis (LPX,  $\text{H}_2\text{O}_2$ , SOD, CAT, GPX, GR and GST). For *in vitro* studies tissue was homogenized in 1.15% ice-cold KCl solution. Homogenization and centrifugation process were similar to those mentioned above.

During *in vitro* condition supernatant fractions of digestive gland of untreated mussels were incubated with various concentrations of freshly prepared solutions of mercury chloride and GSH for 20 min at 37 °C.

#### *Lipid peroxidation measurement*

LPX level was assayed by measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids hydro peroxides were determined by the TBA reaction as described by Ohkawa et al. (1979). The absorbance was read at 532 nm after removal of any fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (Wills, 1969) and expressed as nmol TBARS formed (mg protein)<sup>-1</sup>.

#### *Estimation of Protein carbonyl*

Samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 2 mM EDTA, 0.1% (w / v) digitonin, and 0.46 mM PMSF. Homogenates were centrifuged at 6000g for 15 min at 4 °C and the supernatants were used for the determinations. For carbonyl quantification the reaction with DNPH as described by Mecocci et al. (1999). The carbonyl content was calculated from the absorbance measurement at 366 nm with the use of molar absorption coefficient of 22,000 mol.cm<sup>-1</sup> and expressed as nmol (mg protein)<sup>-1</sup>.

#### *Alkaline unwinding assay*

Alkaline DNA unwinding assay was determined according to Shugart (1988 a b) with slight modification. Approximately 100-150 mg of the tissue was homogenized using 1 ml of 1N NH<sub>4</sub>OH in 0.2% Triton X-100. Each sample was homogenized with 5-10 standardized strokes of the pestle to minimize any potential effect on DNA integrity introduced by the homogenization procedure. The homogenate is transferred to a centrifuge tube containing 2 ml water, 6 ml of Phenol/ Chloroform/ Isoamyl alcohol (PCI) (25/24/1: v/ v/ v) and is allowed to stand for 10-15 min. The homogenate was then centrifuged at 10000g for 20 min at 4 °C. The aqueous layer was transferred to a new microcentrifuge tube. The solution was then digested by 5µl of Ribonuclease A (10 mg ml<sup>-1</sup>) for 30 min at 37 °C and extracted successively by equal volume of PCI. The aqueous layer was transferred to a new microcentrifuge tube. The DNA was recovered by Sephadex-G-50 column

(1cm i.d: 4 ml settled bed volume), equilibrated in G-50 buffer (0.15 M NaCl, 0.01 M Tris pH 7.4, 1 mM MgCl<sub>2</sub>, 5 mM EDTA).

The DNA sample was diluted and separated into three equal proportions. Each tube contains 100 µl of DNA sample and 50 µl of 0.05 N NaOH. The contents were mixed and maintained at 4 °C, 80 °C and 38 °C for 25 min, for fluorescence determination of double-stranded DNA (ds DNA), single-stranded DNA (ss DNA) and partially unwound DNA (au DNA). After incubation sample was neutralized by addition of 50 µl of 0.05 N HCl. This is followed immediately by the addition of 5 µl of 0.2% SDS in 2 mM EDTA, 3 ml of 0.2 M potassium phosphate buffer (pH 6.9) and 1µl of (mg ml<sup>-1</sup>) Hoechst dye 33258. The contents were mixed and react in darkness for 15 min to allow fluorescence to stabilize. The fluorescence of ds DNA, ssDNA, and auDNA were measured, using a spectrofluorometer (Shimadzu RF-501) [Ex: 360nm, Em: 450nm]. Fluorescent values were used for the determination of the F value = (au DNA – ss DNA) / (ds DNA- ss DNA).

#### *Antioxidant enzymes*

The activity of total SOD determined by the method of Paoletti et al. (1990) by monitoring rate of oxidation of NADH. One unit of SOD activity was defined as 50% inhibition of oxidation of NADH in the reaction. The absorbance was recorded at 340 nm and expressed as U (mg protein)<sup>-1</sup>. CAT activity was determined according to Aebi (1974) by monitoring decrease in absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm and enzyme activity was expressed as nkat (mg protein)<sup>-1</sup> (1kat=1 mol sec<sup>-1</sup>). The activity of GPX was determined by the method of Paglia and Valentine (1967) using cumenehydroperoxide as the substrate and enzyme activity was monitored by recording oxidation of NADPH at 340 nm. Enzyme activity was expressed as nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup> using a molar extinction coefficient of 6.22 mM<sup>-1</sup> cm<sup>-1</sup>. The activity of GR was assayed following the rate of NADPH oxidation at 340 nm in the presence of GSSG (Massey and Williams, 1965). Enzyme activity was expressed as nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup> using a molar extinction coefficient of 6.22 mM<sup>-1</sup> cm<sup>-1</sup>. GST activity was measured according to Habig et al. (1974) using CDNB as a substrate. The change in the absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed min<sup>-1</sup> (mg protein)<sup>-1</sup> using a molar extinction coefficient of 9.6 mM<sup>-1</sup> cm<sup>-1</sup>.

### *Measurement of small antioxidant molecules*

The digestive gland was homogenized in 5% (w/v) TCA in an ice bath. The homogenates were centrifuged at 1000g for 30 min, and the deproteinised supernatant was used for the estimation of GSH (Ellman, 1959) and ASA (Mitusi and Ohata, 1961).

### *Hydrogen peroxide*

H<sub>2</sub>O<sub>2</sub> content in post-mitochondrial fractions was determined spectrophotometrically according to the methods of Pick and Keisari (1981) using HRP and phenol red.

### *Metallothionein and protein determination*

MT content was determined according to the method of Viarengo et al. (1997) that was modified by Petrovic et al. (2001). Tissue homogenate was prepared by 20 mM Tris-HCl buffer pH 8.6, containing 0.5 mM PMSF and 0.01% β-mercaptoethanol. The homogenate was then centrifuged at 10000g for 30 min at 4 °C to obtain a supernatant containing MT. Aliquots of 1 ml supernatant were added with 1.05 ml of cold (-20 °C) absolute ethanol and 80 µl of chloroform. The samples are then centrifuged at 6000g for 10 min at 4 °C. The supernatant was combined with three volumes of cold ethanol (-20 °C), maintained at -20 °C for 1 hr and centrifuged at 6000g for 10 min. The pellet was washed with 87% ethanol and 1% chloroform solution. Finally pellet was dissolved in 5 mM Tris-HCl pH 8.6 and 1 mM EDTA. The MT content in the pellet was evaluated using 0.6 mM DTNB buffered with phosphate buffer pH 8.0. MT concentration was estimated utilizing GSH as standard and expressed as nmol of GSH (mg protein)<sup>-1</sup>.

Protein content was estimated by the Folin-Phenol reaction as described by Lowry et al. (1951) using bovine serum albumin as standard.

### *Statistical analysis*

Results are expressed as mean ± standard deviation (SD). Difference among the means of control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when P < 0.05.



### 3. Results

#### *LPX.*

The results on the *in vitro* effects of incubation of various mercury chloride concentrations on LPX content in digestive gland are shown in Fig-1. An increase in TBARS content to 19%, 16% and 42.36% in digestive gland were observed in response to 1, 10 and 100  $\mu\text{M}$  of  $\text{HgCl}_2$  exposures. Addition of GSH to the reaction mixture showed a reduction in TBARS to 8%, 16% and 35.3% in digestive gland. The digestive gland also showed an increased TBARS (Fig-2A,  $P < 0.05$ ) level on 5<sup>th</sup> and 10<sup>th</sup> d, which decreased substantially on 15<sup>th</sup> d of Hg exposure ( $P < 0.001$ ).

#### *PC, DNA strand break and $\text{H}_2\text{O}_2$ .*

Similarly PC showed elevated values throughout the experimental period in mercury treated groups (Fig-2B,  $P < 0.05$ ). Genotoxic effects in the form of F-values did not show any changes on the 5<sup>th</sup> and 15<sup>th</sup> d but a significant decrease was seen on 10<sup>th</sup> day in the treated specimen (Fig-2C,  $P < 0.05$ ). The  $\text{H}_2\text{O}_2$  level showed a significant increase throughout the experiment in relation to control (Fig-2D,  $P < 0.05$ ).

#### *Antioxidant enzymes*

Determination of antioxidant status of mussels exposed to metals is important to understand the toxic mechanism and predict the damage potential in the organism. An increased oxidative stress has been suggested to enhance the antioxidant enzyme activity in animals, as a protective action towards the oxidative stress (Halliwell and Gutteridge, 2001). SOD activity decreased on 5<sup>th</sup> d and increased substantially on 15<sup>th</sup> d of Hg exposure indicating protective behaviour of the cell against superoxide radicals (Fig-2E). Reduced SOD activity seen initially in the Hg mussels may suggest enzyme inhibition by ROS (Hodgson and Fridovich, 1975). Activities of CAT and GPX were almost similar to control during first 10 days, but increased on 15<sup>th</sup> d (Fig-2F, 3 A,  $P < 0.05$ ). GR is vital for maintaining the thiol status of the cell through regeneration of GSH from oxidized glutathione (GSSG). Hence GR induction becomes important during stress conditions, which is evidently seen in the present experiments. GR activity significantly increased on the 5<sup>th</sup> and 15<sup>th</sup> d of Hg exposed mussels (Fig-3B,  $P < 0.05$ ) indicating its role in detoxification mechanism. GSTs are involved in the detoxification of both reactive intermediates and oxygen radicals (Van der Oost et

al., 2003). Induction of GST activity in mussels is stimulated by large variety of pollutants including metals (Prakash and Jagannatha Rao, 1995) and petroleum aromatic hydrocarbons (Pennec and Pennec, 2003). In the present study GST, which was similar to control on 5<sup>th</sup> d, progressively increased in Hg stressed mussels from 10<sup>th</sup> d onwards (Fig-3C) indicating activation of detoxification mechanism.

#### *Non-enzymatic antioxidants*

GSH showed increased values in relation to control from 10<sup>th</sup> day onwards indicating their protective action against the oxidative stress generated by Hg (Fig-2 D, P < 0.05). MT level was significantly high on 5<sup>th</sup> and 10<sup>th</sup> day of the metal exposure but remained at par with control on the last day of the experiment (Fig-2E, P < 0.05). The higher MT value suggests its active role in protecting the cell from metal toxicity. ASA which was comparable to control initially showed increased values on the 15 d Hg exposure (Fig-2F, P < 0.05).

#### **4. Discussion**

Laboratory studies on analysis of stress responses in tissues of organism exposed to metal can help to understand mechanism through which metals exert their toxicity in organisms and hence the results can be used to explain the impact of heavy metal toxicity on organisms in fields. It is obvious from the present study that exposure of mussels to sub-lethal concentration of mercury not only influence the oxidative stress parameters but also the antioxidant enzymes and other products in digestive gland of *P. viridis*.

An enhanced level of TBARS in digestive gland of the mussel in response to mercury suggests that the animals are under oxidative stress. These results are in agreement with those reported by other authors (Geret et al., 2002; Almeida et al., 2004). Organisms are equipped with a cascade of enzymes to counteract ROS produced either during normal metabolism or due to exposure of xenobiotics or metal ions (Halliwell and Gutteridge, 2001). In this, SOD is the first one which dismutates  $O_2^{\cdot-}$  into  $H_2O_2$ , which is further scavenged by CAT and GPX. Hence the initial decreased SOD activity seen in the digestive gland of the Hg exposed mussels in the present experiment may be indicative of increased  $O_2^{\cdot-}$  radicals. These oxyradicals in combination with  $H_2O_2$  may give rise to the production of hydroxyl radicals by classic Haber-Weiss reaction resulting

in elevated TBARS and PC contents. Increase in antioxidant enzymes such as CAT, GPX, GR and GST in tissue specific manner may indicate the adaptive approach by the animal to counteract oxidative stress, generated as a consequence of mercury exposure. PC induction, which was seen throughout the experimental period in the Hg exposed specimen, agrees well with the observations of Labieniec and Gabrylak (2004) and Parvez and Raisuddin (2005). These authors have shown that mussels and fishes exposed to tannins and metals increased PC content in the animals. Hg ions are known to induce oxidative stress by triggering ROS through mitochondrial electron transport chain in the animals (Lund et al., 1991; Pourahmad et al., 2003). This process could give rise to the oxidation of proteins. It is well known that carbonyl compounds are toxic due to their carcinogenic properties. Their presence in the environment is therefore a great concern as regard to their adverse effects to health (Bakeas et al., 2003). It is for the first time that PC induction due to mercury toxicity has been recorded in the digestive gland of *P. viridis*, which could act as a marker for mercury contamination.

DNA which is another target biomolecule in cellular oxidative injury shows base pairs damages, strand cross-links and strand breaks as a result of free radical exposure (Dizdaroglu et al., 2002). DNA strand break also depends on toxicant uptake, distribution, storage, biotransformation, elimination (Black et al., 1996) as well as repair mechanism (Ching et al., 2001). The F values which were almost equal to control on 5<sup>th</sup> and 15<sup>th</sup> day showed a decrease on 10<sup>th</sup> day, giving the signs of toxicity and repair mechanism in the Hg exposed animals. Similar responses have been reported in relation to benzo[a]pyrene and Aroclor 1254 exposures in *Perna viridis* by Siu et al., (2003). In the present case may be the result of activation DNA repair mechanism in the animals (Siu et al., 2003) or activation of antioxidant defence systems, which scavenges free radicals produced during Hg exposure or due to the activation of DNA repair mechanism in the animals.

Changes in SOD content with initial low and increase on 15<sup>th</sup> day in exposed animals could be either due to its inhibition or over utilization to overcome Hg toxicity. The intense metabolic transformation that takes place in digestive gland, at initial stage of mercury exposure, increases the ROS production such as H<sub>2</sub>O<sub>2</sub>, which could be responsible to keep the SOD enzyme low on 5<sup>th</sup> day. Alternatively initial decreased SOD activity could be a part of mercury toxicity response, in which other antioxidants play an active role to overcome ROS toxicity, thereby keeping SOD low. But the increased SOD seen on 15<sup>th</sup> d could suggest its active involvement in dismutation of O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub>.

Increase in SOD activity has been reported by Prakash and Jagannatha Rao (1995) in mussels exposed to Al, Pb and Cd. However the unchanged behavior of this enzyme was observed by Doyotte et al. (1997) in his experiment on exposure of mussel *U.temidis* to copper. Similar results were also reported 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 compared to the control. Since the detailed mechanism of action of Hg on SOD activity is not fully understood, further studies would be needed to explain this process in details.

The principal H<sub>2</sub>O<sub>2</sub> scavenging enzymes are CAT and GPX. Although these enzymes did not show much changes initially in relation to control, a significant increase recorded on the 15<sup>th</sup> day may be due to continuous production of O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub> in digestive gland as a result of Hg toxicity. Consequently this reactive oxygen could stimulate its scavenging mechanism activated by CAT and GPX production, which is evident in higher enzyme values on 15<sup>th</sup> day. These results are consistent with other studies in which increased CAT (Prakash and Jagannath Rao, 1995) and GPX activities are reported in digestive gland of *P. viridis* in response to metals, seasonal changes in environmental parameters and pollutants (Torres et al., 2002; Verlecar et al., 2007, 2008).

Significant increase in GST activity from 10<sup>th</sup> day onwards seen in Hg exposed specimen suggests the protective action against reactive oxygen radicals. Induction of this enzyme has also been reported in crabs exposed to Hg (Elumalai et al., 2007) and in *Mytella guyanensis* collected from heavy metals contaminated sites (Torres et al., 2002), indicating that GST activity in the digestive gland could be a good indicator of Hg exposure.

The higher GR activity observed presently could be the part of the protective response in the digestive gland to maintain the intracellular thiol status. In agreement with our results Lash and Zalups (1996) have also reported increased GR activity in rat renal tissue under in vivo Hg stress. A significant positive correlation between GR and GSH seen presently also supports that enzyme is involved reducing the oxidative stress ( $r = 0.571$ ,  $P < 0.05$ ). GSH functions by reacting with O<sub>2</sub><sup>•-</sup>, peroxy radicals (ROO<sup>•</sup>) and singlet oxygen (<sup>1</sup>O<sub>2</sub>) followed by the formation of GSSH and other disulfides (Meister, 1988). Significant increase in GSH content from 10<sup>th</sup> day onwards, suggests that GSH accumulation may help counteract oxidative damage caused by Hg in *P.viridis*. Interestingly, addition of GSH to the reaction mixture was able to reduce TBARS level (Fig-1). This indicates that

GSH has protective action, which is known to bind heavy metal ions through its SH group, giving rise to metal-SG complex (Rabestein et al., 1985). Present studies are in agreement with that of Naganuma et al. (1990) which suggest that GSH can act as a physiological defense against mercury-induced cytotoxicity. Induction of GSH in bivalves exposed to Hg has also been reported by Yan et al. (1997). Higher level of GSH along with GST, GPX and GR suggests that the regulation of the ROS, generated due to Hg toxicity in *P.viridis*, is efficiently achieved using the glutathione pathway.

Higher MT content observed in Hg exposed mussels indicates that this protein also has active role in detoxification process by eliminating ROS and metal. Our results are consistent with those of Viarengo et al. (2000), Hamza-Chaffai et al. (2000), Geret, et al. (2002) and Marie et al. 2006, who have also observed higher MT content in bivalves exposed to metals. It has been reported that MT acts as component of antioxidant system and protects cell against cytotoxic effects of ROS (Viarengo et al., 2000). Hence MT induction in *P. viridis* exposed to Hg may be an adaptive mechanism, against Hg generated toxicity.

Ascorbic acid is known to provide an antimutagenic property against a wide variety of chemicals (Sweetman et al., 1997). Due to its nucleophilic properties it binds with mercury ions and reduces Hg induced DNA damage (Rao et al., 2001). In addition, it performs various important physiological functions such as collagen synthesis and formation of connective tissues (Wilson and Poe, 1973). Increased ASA content on the last day of the experiment without any significant change initially, in relation to control, suggests additional availability of GSH on 15<sup>th</sup> day, which may help in reducing dehydroascorbate to ascorbate (Winkler et al., 1994). Further studies are required to understand exact mechanism of ASA involved during Hg exposure.

In conclusion it may be inferred that the significant induction of LPX, PC, DNA damage and H<sub>2</sub>O<sub>2</sub> level in response to Hg exposure suggests the toxic nature of this metal to native *P.viridis*. Positive responses accompanied by elevated activity of GSH, ASA, MT, SOD, CAT, GST, GR and GPX in the Hg exposed specimen indicates the strong potential of animal to protect against the oxidative stress generated by the metal. These responses could be effectively serve as oxidative stress indices to be used as biomarkers in aquatic contamination.

## Acknowledgement

The authors are grateful to the Director of NIO for providing facilities. This work is carried under the funds from NIO Project SSP1738. We are thankful to the Head, Department of Zoology and Biotechnology, Utkal University, for help

## References

Aebi, H., 1974. Catalase In: Bergmeyer, H.U (Eds.), Methods of Enzymatic Analysis. vol.2. Academic Press, New York, pp. 673-678.

Almeida, E. A., Miyamoto, S., Bainy, A.C.D., de Medeiros, M.H.G., Mascio, P.D., 2004. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. Mar. Pollut. Bull. 49, 386-392.

Bakeas, E.B., Argyris, D.I., Siskos, P.A., 2003. Carbonyl compounds in the urban environment of Athens, Greece. Chemosphere 52, 805–813.

Black, M.C., Ferrell, J.R., Horning, R.C., Martin Jr, L.K., 1996. DNA strand breakage in fresh water mussels (*Anodonta grandis*) exposed to lead in the laboratory and field. Environ. Toxicol. Chem. 15, 802-808.

Burlando, B., Bonomo, M., Capria, F., Mancinelli, G., Ponsa, G., Viarengo, A., 2004. Different effects of Hg<sup>2+</sup> and Cu<sup>2+</sup> on mussel (*Mytilus galloprovincialis*) plasma membrane Ca<sup>2+</sup>-ATPase: Hg<sup>2+</sup> induction of protein expression. Comp. Biochem. Physiol. 139, 201–207.

Ching, E.W. K., Siu, W.H. L., Lam, P.K.S., Xu, L., Zhang, Y., Richardson, B. J., Wu, R.S.S., 2001. DNA adduct formation and DNA strand breaks in green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose-and time-dependent relationships. Mar.Pollut. Bull.42,603-610.

Das, S., Patro, S.K., Sahu, B.K., 2001. Variation of residual mercury in penaeid prawns from Rushikulya Estuary, east coast of India. Ind.J..Mar.Sci. 30, 33-37.

Dizdaroglu, M., Jaruga, P., Birincioglu, M., Rodrigue, H., 2002. Free radical-induced damage to DNA: mechanisms and measurement. Free Rad. Biol. Med. 32, 1102-1115.

Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation relevant biomarkers of experimental or field exposure in gills and the digestive gland of the fresh water bivalve *Unio tumidis*. *Aquat. Toxicol.* 39, 93-110.

Ellman, G.L., 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82, 70-77.

Elumalai, M., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. *Chemosphere* 66, 1249-1255.

EPA, 1997. Mercury Study Report to Congress. U.S. Environmental Protection Agency, Washington.

Filho, W.D., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A. and Magalhaes, A.R.M. (2001). Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture* 203:149-158.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. *Fish & Shellfish Immunol.* 16, 501-512.

Geret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). *Aquat. Liv. Res.* 15, 61-66.

Gregory, M.A., Marshall, D.J., George, R.C., Anandraj, A., McClurg, T.P., 2002. Correlations between metal uptake in the soft tissue of *Perna perna* and gill filament pathology after exposure to mercury. *Mar. Pollut. Bull.* 45, 114-125.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione- S-Transferases. The first enzymatic step in mercapturic formation. *J. Biol. Chem.* 249, 7130-7139.

Halliwell, B., Gutteridge, J.M.C., 1985. *Free Radicals in Biology and Medicine*, Oxford-University Press, New York.

Hamza-Chaffai, A., Amiard, J.C., Pellerin, J., Joux, L., Berthet, B., 2000. The potential use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure. *Comp. Biochem. Physiol. C* 127, 185-197.

Heaton, A.C.P., Rugh, C.L., Wang, N.J., 2005. Physiological Responses of Transgenic *merA*-tobacco (*Nicotiana tabacum*) to Foliar and Root Mercury Exposure. *Water Air Soil Pollut.* 161,137–155.

Hodgson, E.K., Fridovich, I., 1975. The Interaction of Bovine Erythrocyte Superoxide dismutase with Hydrogen Peroxide: Chemiluminescence and Peroxidation? *Biochemistry*, 14, 5294-5299.

Jian-bo, S., Lianga, L., Jianga, G., Jina, X., 2005. The speciation and bioavailability of mercury in sediments of Haihe River, China. *Environ. Int.* 31, 357–363.

Karunasagar, D., Balarama Krishna, M.V., Anjaneyulu, Y., Arunachalam, J., 2006. Studies of mercury pollution in a lake due to a thermometer factory situated in a tourist resort: Kodaikkanal, India. *Environ. Pollut.* 143, 153-158.

Kaladharan, P., Pillai V.K., Nandakumar A., Krishnakumar, P.K., 1999. Mercury in seawater along the west coast of India. *Indian J. Mar.Sci.* 28, 338-340.

Labieniec, M., Gabryelak T., 2004. Response of DNA, proteins and membrane bilayer in the digestive gland cells of freshwater mussel *Unio tumidus* to tannins exposure. *Toxicol. in Vitro* 18, 773–781.

Lakshmanan, P.T., Nambisan, P. N. K., 1989. Bioaccumulation and Depuration of Some Trace Metals in the Mussel, *Perna viridis* (Linnaeus). *Bull. Environ. Contam. Toxicol.* 43, 131-138.

Lash, L.H., Zalups, R.K., 1996. Alterations in renal cellular glutathione metabolism after in vivo administration of a subtoxic dose of mercuric chloride. *J. Biochem. Toxicol.* 11, 1–9.

Leonard, S.S., Harris, G.K., Shi, X., 2004. Metal-induced oxidative stress and signal transduction, *Free Radic. Biol. Med.* 37, 1921–1942.

Lowry, O.H., Rosebrough, N.J., Farr, A L., Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Lund, B., Miller, D.M., Woods, J.S., 1991. Mercury-induced H<sub>2</sub>O<sub>2</sub> production and lipid peroxidation in vitro in rat kidney mitochondria. *Biochem. Pharmacol.* 42, 181-187.



Manduzio, H., Rocher, B., Durand, F., Galap, C., Leboulenger, F., 2005. The point about oxidative stress in mollusks. ISJ.2, 91-104.

Marie, V., Baudrimont, M., Boudou, A., 2006. Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradient. Chemosphere 65, 609-617.

Massey, V., Williams, C.H., 1965. On the reaction mechanism of yeast glutathione reductase. Jour. Biol. Chem. 240, 4470-4481.

Mecocci, P., Fano, G., Fulle, S., MacGarvey, U., Shinobu, L., Polidori, M.C., Cherubini, A., Vecchiet, J., Senin, U., Beal, M.F., 1999. Age-dependent increases in oxidative damage to DNA, lipids and proteins in human skeletal muscle. Free Rad. Biol. Med. 26, 303-308.

Meister, A., 1988. Glutathione metabolism and its selective modification, J.Biol. Chem. 263, 7205-7208.

Mitusi, A. and Ohata, T. (1961). Photooxidative consumption and photoreductive formation of ascorbic acid in green leaves. Plant Cell Physiol. 2: 31-44.

Nanda, S., 1993. The environmental impact of a chloro-alkali factory in a river basin in Eastern India. Environmentalist 13, 121-124.

Naganuma, A., Anderson, M., Meister, A., 1990. Cellular glutathione as a determinant of sensitivity to mercury chloride toxicity. Prevention of toxicity by giving glutathione monoester. Biochem. Pharmacol. 40, 693-697.

Ohkawa, H., Ohisi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Paglia, D.E., Valentine, W.N., 1967. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med, 70, 158-169.

Paoletti, F., Mocali, A., Aldinucci, D., 1990. Superoxide-driven NAD(P)H oxidation induced by EDTA-Manganese complex and mercaptoethanol. Chem-Biol. Interact. 76, 3-18.

Parveza, S., Raisuddin, S., 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). Environ. Toxicol. Pharmacol. 20, 112–117.

Pattnaik S., Chainy, G.B.N., Jena, J.K., 2007. Characterization of Ca<sup>2+</sup>-ATPase activity in gill microsomes of freshwater mussel, *Lamellidens marginalis* (Lamarck) and heavy metal modulations. Aquaculture, 270, 443-450.

Pennec, G.L., Pennec M.L., 2003. Induction of glutathione-S-transferases in primary cultured digestive gland acini from the mollusk bivalve *Pecten maximus* (L.): application of a new cellular model in biomonitoring studies. Aquat. Toxicol. 64, 131-142.

Petrovic, S., Ozretic, B., Krajnovic-Ozretic, M., Bobinac, D., 2001. Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. Mar. Pollut. Bull. 42, 1373-1378.

Pick, E., Keisari, Y., 1981. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages. Induction by multiple nonphagocytic stimuli. Cell Immunol. 59, 301-318.

Pourahmad, J., O'Brien, P.J., Jokar, F., Daraei, B., 2003. Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes. Toxicol. in Vitro, 17, 803-810.

Prakash, N.T., Jagannatha Rao, K.S., 1995. Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. Mol. Cell. Biochem. 146, 107-113.

Rabestein, D. L., Guevremont, R., Evans, C.A., 1985. Glutathione and its metal-complexes. In: Siegal, H. (Ed.), Metal Ions in Biological Systems. Marcel Dekker, New York, pp 104-141.

Rajathy, S., 1997. Mercury in water, sediment and in some estuarine organisms of the Ennore Estuary, Madras, Tamil Nadu. J. Mar. Biol. Assoc. India. 39, 174-177,.

Rao, M.V., Chinoy, N.J., Suthar, M.B., Rajvanshi, M.I., 2001. Role of ascorbic acid on mercuric chloride-induced genotoxicity in human blood cultures. Toxicology in Vitro 15, 649 - 654.

Shaw, B.P., Sahu, A., Panigrahy, A.K., 1986. Mercury in plants, soil, and water from caustic chlorine industry. Bull. Environ. Contam. Toxicol. 36, 299-305.

Shugart, L.R., 1988a. An alkaline unwinding assay for the detection of DNA damage in aquatic organisms. *Mar. Environ. Res.* 24, 321-325.

Shugart, L.R., 1988b. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. *Aquat. Toxicol.* 13, 43-52.

Siu, W.H.L., Hung, C.L.H., Wong, H.L., Richardson, B.J., Lam, P.K.S., 2003. Exposure and time dependent DNA strand breakage in hepatopancreas of green-lipped mussels (*Perna viridis*) exposed to Aroclor 1254, and mixtures of B[a]P and Aroclor 1254. *Mar. Pollut. Bull.* 46,1285-1293.

Sweetman, S.F., Strain, J.J., McKelvey-Martin, V.J., 1997. Effect of antioxidant vitamin supplementation on DNA damage and repair in human lymphoblastoid cells. *Nutrition and Cancer* 27,122-130.

Tomiyasu, T., Matsuyama, A., Eguchi, T., Fuchigami, Y., Oki, K., Horvat, M., Rajar, R., Akagi, H., 2006. Spatial variations of mercury in sediment of Minamata Bay, Japan. *Sci. Total Environ.* 368, 283-290.

Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R., Almeida, E.A., Mascio, P.D., Filho, D.W., 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. *Mar. Pollut. Bull.* 44, 923-932.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13, 57-149.

Verlecar, X.N., Jena, K.B. and Chainy, G.B.N. (2007). Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature, *Chem. Biol. Interact.* 167: 219-226.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2008. Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea. *Estuar. Coast. Shelf Sci.* 76, 745-752.

Viarengo, A., Ponzano, E., Donder, F., Fabbri, R., 1997. A simple spectrophotometric method of metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks. *Mar. Env.Res.* 44, 69-84.

Viarengo, A., Burlando., Caratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneine: A comparative overview. *Cell Mol. Biol.* 46, 407-417.

Wilson, R.P., Poe, W.E., 1973. Impaired collagen formation in scorbutic channel catfish. *J. Nutr.* 103, 1359-1364.

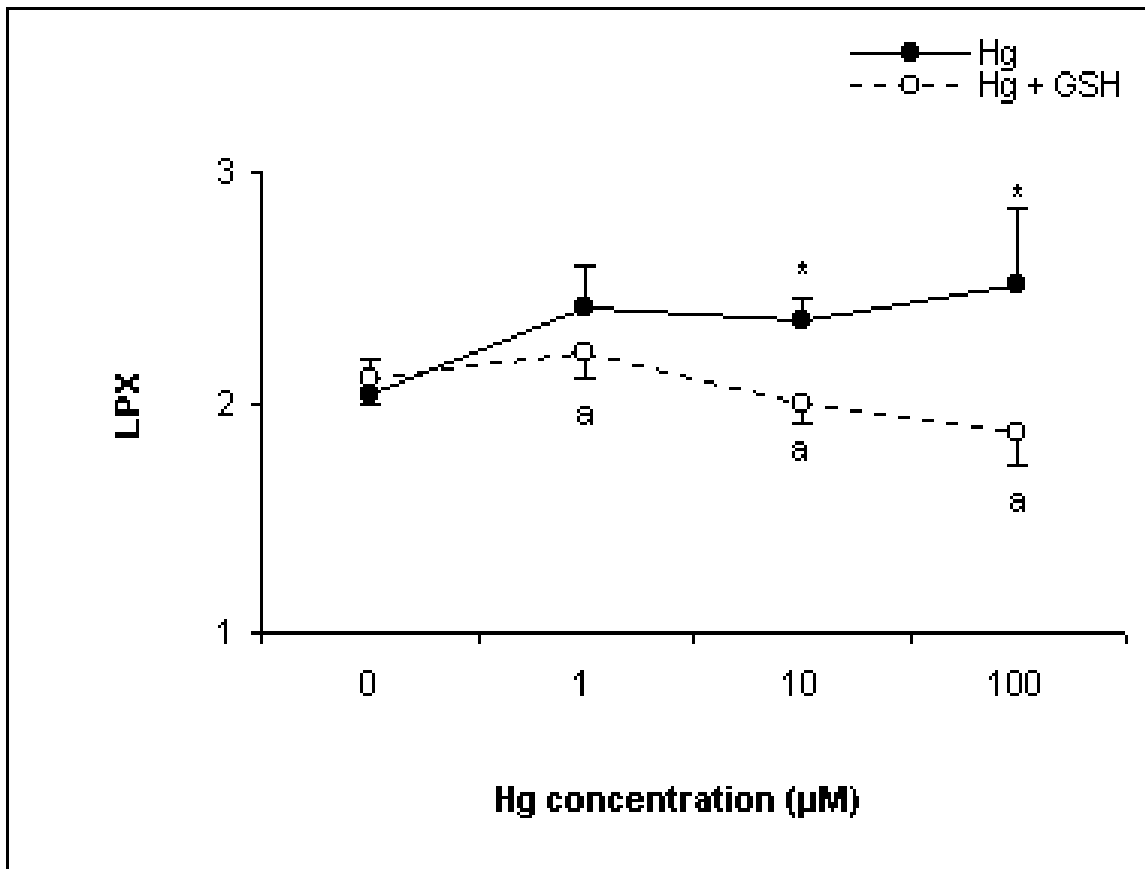
Wills, E.D., 1969. Lipid peroxide formation in microsomes: General Considerations. *Biochem. J.* 113, 315-324.

Winkler, B.S., Orselli, S.M., Rex, T.S., 1994. The redox couple between glutathione and ascorbic acid: A chemical and physiological perspective. *Free Rad. Biol. Med.* 17, 333 - 349.

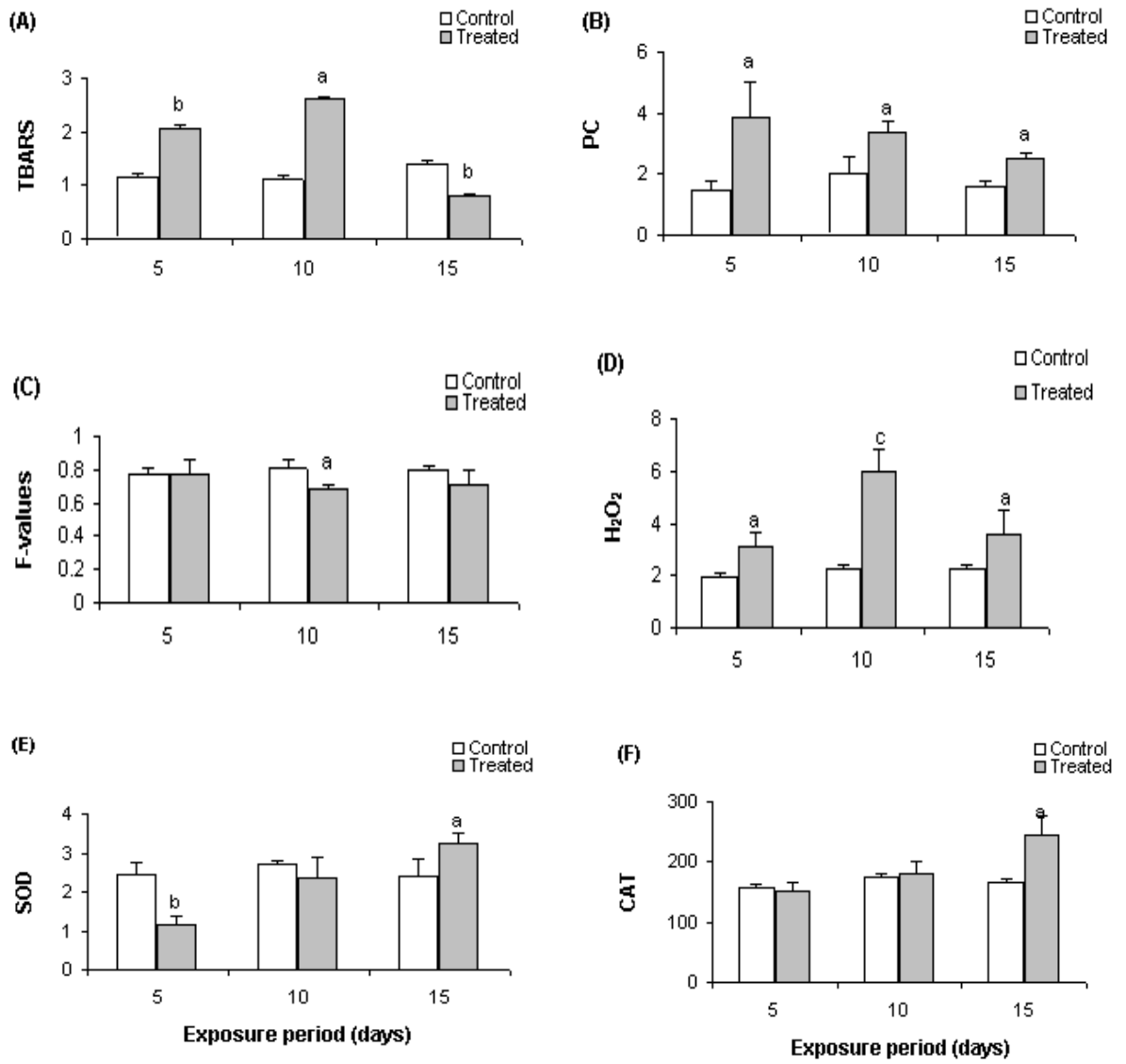
Yan, T., Teo, L.H., Sin, Y.M., 1997. Effect of mercury and lead on tissue glutathione of green mussel, *Perna viridis* (L). *Bull. Environ. Contam. Toxicol.* 58, 845-850.

Yap, C.K., Tan, S.G. Ismail, A., Omar, H., 2004. Allozyme polymorphisms and heavy metal levels in the green-lipped mussel *Perna viridis* (Linnaeus) collected from contaminated and uncontaminated sites in Malaysia. *Environ. Int.* 30,39-46.

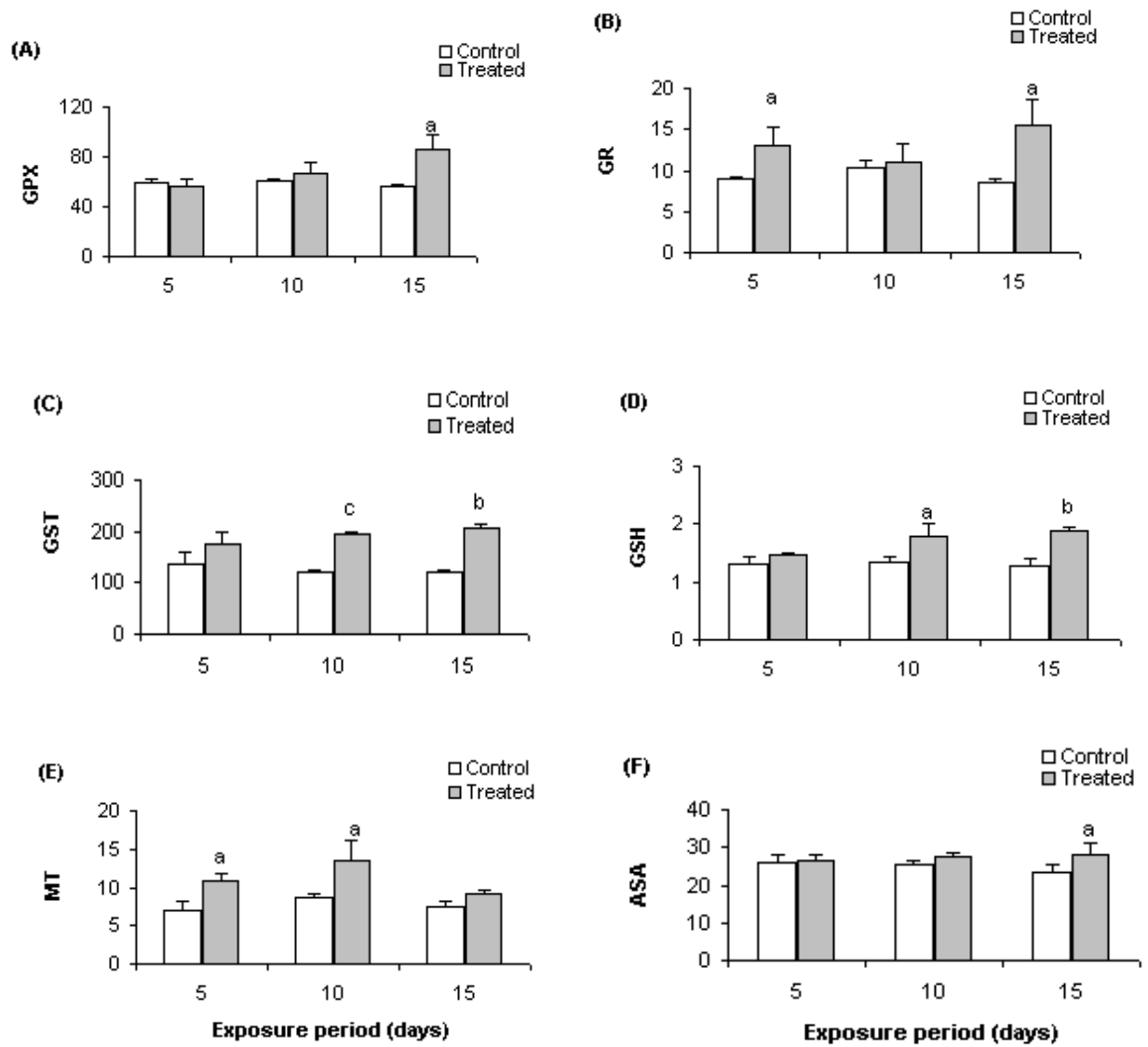
Fig-1



**Fig-2**



**Fig-3**



## Legends to Figures:

**Fig 1 :** In vitro effect of Hg concentrations ( $\mu\text{M}$ ) alone and after supplementing with reduced glutathione on LPX ( nmol TBARS / mg protein). Data are expressed as mean  $\pm$  sd (n=3). (1) Denote indicates significant difference between control and Hg exposed samples \*  $P < 0.05$ . (2) Denote indicates significant difference between glutathione supplementation and respective Hg exposed samples <sup>a</sup> $p < 0.05$ .

**Fig-2** :-Effect of mercury chloride on (A) Lipid peroxidation level (nmol TBARS / mg protein), (B) Protein carbonyl content (nmol / mg protein), (C) DNA damage (F-values), (D)  $\text{H}_2\text{O}_2$  content (nmol / mg protein), (E) SOD activity (Unit / mg protein), (F) Catalase activity (nkat / mg protein) in digestive gland of *P.viridis*, exposed to mercury. Data are expressed as mean  $\pm$  sd (n=3). Denote indicates significant difference between control and Hg exposed mussels <sup>a</sup> $p < 0.05$  . <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$

**Fig-3** :-Effect of mercury on (A) Glutathion peroxidase activity (nmol NADPH oxidized/ min/ mg protein), (B) Glutathione reductase activity (nmol NADPH oxidized/ min/ mg protein), (C) Glutathione- S-Transferase activity (nmol CDNB conjugate formed/ min/ mg protein), (D) Reduced glutathione level ( $\mu\text{mol}$  GSH/ g wt. tissue), (E) Metallothionein content (nmol GSH/ mg protein) and (F) Ascorbic acid content ( $\mu\text{g}$  ASA / g wt. tissue) in digestive gland of *P. viridis*, exposed to mercury. Data are expressed as mean  $\pm$  sd (n=3). Denote indicates significant difference between control and Hg exposed mussels <sup>a</sup> $p < 0.05$  . <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$