

Antioxidant responses in gills and digestive gland of oyster *Crassostrea madrasensis* (Preston) under lead exposure

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Abstract

Crassostrea are ecologically and economically important bivalves and provide a good livelihood for coastal regions of many countries, including India. This study aims at evaluating the response of the antioxidant defense system in oyster *Crassostrea madrasensis* against lead (Pb) exposure under laboratory conditions. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST) and oxidative damage parameter lipid peroxidation (LPO) were measured in the gills and digestive glands of oysters exposed to 1–50 µg/l of Pb (NO₃)₂ over a period of 8 days. LPO index increased progressively with increase in Pb concentration (1, 10, 25 and 50 µg/l) in both tissues, gills and digestive gland. Although CAT and SOD activities induced together in the initial phase (upto 6th day), their activities decreased at a later stage of the experiment. However, GST activity increased on 8th day in both the tissues at concentration 10, 25 and 50 µg/l indicates the compensatory defense mechanism against oxidative stress. The induced antioxidant responses recorded at 25 and 50 µg/l of Pb concentrations suggest the presence of Pb-induced oxidative stress at these concentrations. The results of this work also indicate that LPO, SOD, and GST could be used as biomarkers to assess the impact of Pb on the *C. madrasensis*. This study concludes that any high level of dissolved Pb concentration (>10 µg/l) in surrounding seawater could be harmful to the physiology of the *C. madrasensis*.

Keywords *Crassostrea*, Lead, Bioaccumulation, Oxidative stress, Antioxidant enzymes, Biomarker

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Introduction

Oysters (Mollusca: Bivalvia) are sedentary, known to bioaccumulate and tolerate high concentrations of metals (several magnitudes higher than their ambient concentrations). Therefore, worldwide they are used extensively as a biomonitor for metal pollution and eco-toxicological studies (O'Connor, 2001; Funes et al., 2006; Alfonso et al., 2013). Oysters are popular worldwide due to their high nutritional values (Asha et al., 2014). In India, 2400 tonnes of oyster production was recorded from the Indian coast in the year 2008 (http://www.fao.org/fishery/countrysector/naso_india/en).

Lead (Pb) is ubiquitous in presence and one of the most toxic element occurs in almost all the ecosystem (Hsu and Guo, 2002; Flora et al., 2012). It is derived from both natural sources such as weathered (dissolved) or eroded (particulate) earth's surface, volcanic activities, atmospheric aerosol, biogenic forest fires as well as anthropogenic activities. In recent years, however, increase in various anthropogenic activities such as lead smelting, use of lead based-antifouling paints, ceramics, plastics, leaded gasoline, combustion of fossil fuel have significantly increased the Pb concentration in the aquatic environment (MacFarlane, 2001; Flora et al., 2012). High to moderate levels of Pb content have been found in tissues of edible clam *Paphia malabarica* (average: 30.3 mg/kg) (Krishna Kumari et al., 2006), suspended particulate matter (range: 27.6–91.4 mg/kg) (Kessarkar et al., 2013) and in surface sediments (average: 22.6 mg/kg) (Veerasingam et al., 2015) across the estuarine waters of Goa. Moreover, higher concentrations of Pb level (>1 mg/kg, permissible limit) have been observed in the tissues of edible oyster *Crassostrea* sp. from the coastal waters of Goa (Shenai-Tirodkar et al., 2016). It is recognized that impact of these accumulated metals cannot be judged only by quantification because it does not provide a clear indication of toxic effects of pollutant on the aquatic organism (Livingstone, 2001).

Biochemical biomarkers have been commonly used to measure the effects of contaminants on organisms in ecotoxicological studies (Livingstone, 1993). Under normal metabolic processes, a balance between generation and neutralization of reactive oxygen species (ROS) include superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot HO$) is maintained by organisms. However, it has been proved that bioaccumulated metal ions enhance the formation of reactive radicals in the cells which cause the cell damage (Leonard et al., 2004; Valko et al., 2005). It is well known that exposure to Pb boosts the ROS production, which in turn results in damage to the cell membrane, protein oxidation, lipid peroxidation and DNA damage (Halliwell and Gutteridge, 1989; Gurer and Ercal, 2000; Ercal et al., 2001; Hsu and Guo, 2002; Flora et al., 2012). This oxidative damage can be assessed by measuring thiobarbituric acid reactive substances (TBARS) reflecting the state of lipid peroxidation of the cell membrane (Ohkawa et al., 1979). Further, to

protect against oxidative stress organism possess major antioxidant defense system which includes three main enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST). However, noticeable variation in responses (induction and/or inhibition) of biochemical biomarker were recorded in previous laboratory and field studies which mostly depend on the type and amount of contaminants, type of species, the tissue, and the sampling time, etc. (Zanette et al., 2006; Luna-Acosta et al., 2010; Cotou et al., 2013).

Presently, there is very limited work published with respect to Pb exposure in bivalves (Prakash and Jagannatha Rao, 1995; Yan et al., 1997; Dafre et al., 2004, Fernandez et al., 2010). Particularly, Pb toxicity study on oyster species from Indian coast has not been conducted which is harvested and consumed on large scale and on regular basis. Exposure of oysters to various concentrations of Pb, coming from discharge of dissolved and particulate forms of Pb into the marine ecosystem of Goa coast (India), has necessitated this study to understand the antioxidant defense response of oysters against Pb exposure. It has been proved that field-based effects could well be understood by conducting laboratory exposures of organisms to a particular contaminant (Verlecar et al., 2008).

In this study, gills and digestive glands of oyster, *C. madrasensis*, were examined to assess the effect of Pb exposure using biochemical markers such as LPO, SOD, CAT and GST. The reason for selecting gills and digestive gland is because these tissues form an active site for xenobiotic uptake and oxy-radical generation as well as enzyme biotransformation process (Livingstone et al., 1992).

2. Materials and methods

2.1. Sampling site and oyster collection

A total of 150 individual adult oysters, *C. madrasensis* (60–70 mm), irrespective of sex, were collected during low tide in post-monsoon (October, 2015) from Galgibag estuary, South Goa coast, India. Galgibag site (Lat. 14° 57' 54.66" N Long. 74° 03' 20.16" E) was chosen because this site is located far away from potential pollution sources such as industries and harbor activities (Fig. 1). The hydrological parameters (water temperature: 29±1 °C, pH: 7.9±0.4 and salinity: 27±1 psu) were recorded at the time of oyster collection. The samples were brought to laboratory in seawater and cleaned to remove fouling organisms and debris. All the oysters were acclimatized for 8 days in aerated seawater at room temperature before conducting the acute semi-static renewal Pb toxicity (8 days) experiment (Thompson et al., 2012).

2.2. Experimental design

After the acclimatization period, the oysters were distributed into five different groups (n= 15, each tank) and subjected to five treatments: one seawater control and four different Pb concentrations

(1µg/l, 10µg/l, 25µg/l, and 50 µg/l). These concentrations were selected based on environmental realistic concentration (Shenai-Tirodkar et al., 2016) and concentration large enough to induce biomarker response. Mortality was monitored and dead oysters were removed. Under these conditions, the state of the antioxidant enzyme system was assessed.

The experiment was setup in duplicate in 25 l capacity plastic tanks (~1.5 l of seawater per oyster). Seawater was continuously aerated and standard hydrological parameters (temperature 29±1 °C, pH 8.2±0.4 and salinity 27±1 psu) were recorded throughout the experiment. Lead nitrate [Pb (NO₃)₂] (Merck India Ltd, Mumbai) was used to prepare stock solution (100 mg/l) and preserved in a refrigerator until use. This was treated as main stock solution from which fresh sub-stock solutions were prepared and used for preparation of required concentrations of Pb. All the glassware used during experiment was washed with 10 % HNO₃ (nitric acid) and well rinsed with distilled water. The whole exposure media was renewed on daily basis and replaced with fresh natural seawater, maintaining respective concentrations of Pb (NO₃)₂. Oysters were not fed during experimentation. At each sampling (2nd, 4th, 6th and 8th day of experiment duration), two of the surviving oysters were sacrificed from each treatment tank and immediately gills and digestive gland were dissected for further analysis. All the further analyses were performed in triplicates to rule out the experimental bias.

2.3. Determination of Pb content in gills and digestive gland of *C. madrasensis*

Gills and digestive gland of *C. madrasensis* were processed for Pb estimation according to the method described in Cheung and Wong (1992) with slight modifications. In detail, both the tissues were dried at 60 °C till constant weight was attained, and ground by using agate mortar and pestle. The 0.5 g of dried tissue powder was soaked overnight in 5 ml of 65 % HNO₃. Reaction mixture was heated at 80 °C till dry and re-dissolved in 3 ml of 65 % HNO₃ to achieve the complete digestion of the tissue. The digest was allowed to cool at room temperature. The digested clear solution was then filtered through Whatman No. 42 filter paper and diluted to 10 ml with 2 % HNO₃ used for metal determination. Fish protein certified reference material (DORM-4) was used to ensure the quality of the results. Determination of Pb was made by using Flame Atomic Absorption Spectroscopy (Flame-AAS). Utmost care was taken at every step of processing the sample, to avoid contamination. All reagents used in the analysis were of the purest grade. Appropriate blanks and standards were also prepared by using the same method. Appropriate dilutions of samples were made depending on the sensitivity of detection in these samples. Precision measured as relative standard deviation (% RSD) of triplicate sample values were <10 %. Accuracy of Pb analysis was expressed in recovery percentage 91.35 %.

2.4. Biochemical analysis of gills and digestive gland of *C. madrasensis*

2.4.1. Sample preparation

The gills and digestive gland of *C. madrasensis* were thoroughly washed with phosphate buffer (50 mM; pH 7.4) and homogenized in 50 mM phosphate buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 0.15 M potassium chloride (KCl), 0.01% phenylmethylsulfonyl fluoride (PMSF), pH 7.4). Homogenization was carried out using motor driven Teflon Potter-Elvehjen homogenizer. Further, homogenates were centrifuged at $10000 \times g$ for 30 min. at 4°C. The resulting supernatant fractions were stored at -80 °C until the quantification of the activities of LPO, SOD, CAT, and GST.

2.4.2. Estimation of lipid peroxidation

The LPO index was estimated using the protocol described by Ohkawa et al. (1979) with minor modifications. Briefly, tissue homogenate (0.5 ml) was mixed with 1.5 ml of 0.8 % thiobarbituric acid (TBA) solution, 1.5 ml of 20 % acetic acid (pH 3.5), 0.2 ml of 8.1 % sodium dodecyl sulfate (SDS), 0.2 ml double distilled water and 0.1 ml of 0.76 % butylated hydroxytoluene (BHT). The mixture was heated at 95 °C for 60 min. and cooled at room temperature. After that samples were centrifuged at $5000 \times g$ for 10 min. and the absorbance of the supernatant was measured at 532 nm against blank. The LPO index was calculated by using an extinction coefficient of 1.56×10^5 M/cm (Wills, 1969) and expressed as nmol TBARS formed/mg protein.

2.4.3. Estimation of superoxide dismutase and catalase activity

Superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities were measured by using EnzyChrom superoxide dismutase assay kit (ESOD-100) and EnzyChrom catalase assay kit (ECAT-100) respectively, as per the manufacturer's instructions. Both the kits were bought from Bioassay Systems, USA. Assays were performed in a microplate reader (FLUOstar Omega; BMG LABTECH Pty. Ltd.) by using 96 well plates. The results of SOD were expressed as Unit/mg protein whereas CAT activity was presented as nkat/mg protein.

2.4.4. Estimation of glutathione s-transferase activity

Glutathione-s-transferase (EC 2.5.1.18) activity was determined following the methods of Mannervik (1985) with minor modifications. The reaction mixture contained 950 μ l assay buffer phosphate-buffered saline (PBS) (pH 6.5), 10 μ l of 100 mM glutathione (GSH), 30 μ l sample (200-250 μ g protein) and 10 μ l of 100 mM 2,4- dinitrochlorobenzene (CDNB). The difference in absorbance was measured at 340 nm for every 30 sec. for 5 min. The activity of GST was determined using molar

extinction coefficient of 0.0096/ μ M/cm for CDNB. The results were expressed as nmol CDNB conjugates formed/min/mg protein.

2.4.5. Estimation of total protein concentration

Total protein concentrations of tissue samples were analyzed using bovine serum albumin (BSA) as standard as described by Lowry et al. (1951).

2.5. Statistical analysis

Data were expressed as mean \pm standard deviation (SD) (n = 3). All the data were first checked for normality using Shapiro–Wilk’s test, and homogeneity of variance by using Levene’s test. Data that failed to meet the normal distribution were transformed by log (x+1) transformation prior to statistical testing. Significance of variability among groups with Pb concentrations, exposure time and the tissues were evaluated for each biomarker as well as for the Pb content by using three-way analysis of variance (three-way ANOVA). For the multiple comparison among groups post hoc test (Tukey’s HSD test) was performed. To find out the significant relationship, Pearson correlations were performed among Pb content, oxidative stress and antioxidant response in gills and digestive glands of *C. madrasensis*. Statistical significance of data was measured at $P < 0.05$ level. All the tests were performed using STATISTICA 8 (StatSoft, Inc., USA) software.

3. RESULTS

During acclimatization period, dead oysters were removed from the respective tanks. After acclimatization, during the experimental period, oyster’s mortality was observed only in tanks containing concentration of Pb more than 10 μ g Pb/l. A total of 3 oysters died from 10 μ g Pb/l treated tank on 5th and 7th day. Similarly, 3–4 oysters died at 25 μ g Pb/l and 50 μ g Pb/l treated tank on 4th and 7th day respectively.

3.1. Concentration of Pb in gills and digestive gland of *C. madrasensis*

Concentrations of Pb in gills ranged from 0.27 \pm 0.07 mg/kg to 1.27 \pm 0.01 mg/kg whereas in digestive gland, it varied between 0.1 \pm 0.05 mg/kg and 0.95 \pm 0.05 mg/kg (Fig. 2). On the 8th day, 50 μ g/l treated oysters showed the highest accumulation (1.27 \pm 0.01 mg/kg) in gills which is more than the allowable limit for human consumption according to guidelines set by EU, 2001. Accumulation of Pb in the gills and digestive gland of *C. madrasensis* increased significantly ($p < 0.05$) over a period of days in each Pb treatments as compared to control. Further, significantly higher accumulation of Pb was observed in gills than digestive gland during the experiment at all the exposed concentrations (Table 1).

3.2. Biomarker responses in gills and digestive glands of *C. madrasensis*

Oxidative stress indices such as LPO, SOD, CAT, and GST in gills and digestive gland of oyster have been measured under the semi-static Pb exposure experiment. Considerable changes took place in the oxidative defense system of gills and digestive gland after exposure to different concentration. Results of three-way ANOVA revealed the significant ($p < 0.05$) differences in biomarker responses among variables such as concentrations, exposure time (days), types of tissue and interactions among these variables (Table 2).

3.2.1. Lipid peroxidation (LPO)

LPO index in both gills and digestive gland showed significant ($p < 0.001$) increase against each Pb treatment as compared to respective control. Moreover, LPO index showed strong positive correlation with the Pb content ($p < 0.001$) in gills and digestive gland (Table 3). Although it was observed that continuous oxidative load is experienced by gills, digestive gland exhibited significantly higher oxidative stress compared to gills on 2nd and 6th day (Figs. 3 a and b). Further, the digestive gland of oysters exposed to 50 $\mu\text{g/l}$ showed declined LPO activity significantly from 6th day to 8th day ($p < 0.001$).

3.2.2. Superoxide dismutase (SOD)

Figures (3 c and d) and Table 2 shows that SOD activity in digestive gland was significantly higher than gills. SOD activity in gills and digestive gland from control tank did not show much variation during successive exposure periods. In particular, gills showed significant ($p < 0.001$) increase in SOD activity at 10, 25 and 50 $\mu\text{g/l}$ treated groups until 6th day, which then dropped prominently ($p < 0.001$) on 8th day (except 10 $\mu\text{g/l}$). A similar trend was observed in digestive gland from all the treated groups (except in 50 $\mu\text{g/l}$ treated group where SOD activity significantly increased). Activities of SOD was closely correlated with Pb concentrations and LPO index in both the tissues (Table 3).

3.2.3. Catalase (CAT)

Activity of CAT in gills increased significantly ($p < 0.001$) until the 4th day in 1 and 10 $\mu\text{g Pb/l}$ exposed groups and till the 6th day in 25 and 50 $\mu\text{g/l}$ exposed groups. This was followed by a slight decrease in CAT values on 8th day. Whereas gills from control group showed no significant variation in CAT measurements throughout the experiment. A similar trend of CAT activities was also noticed in digestive gland of treated oysters ($p < 0.001$) (Figs. 3 e and f). The CAT activity showed a positive correlation with Pb concentration, LPO ($p < 0.01$) and GST ($p < 0.05$) in both gills and digestive gland of oyster (Table 3).

3.2.4. Glutathione S-transferases (GST)

Response of GST in gills and digestive gland to Pb exposure are shown in Figs. 3 g and h. Low levels of GST was observed in gills and digestive gland ($p < 0.05$) from all Pb exposed groups compared to controls till the 6th day (except in 50 $\mu\text{g/l}$). However, on the 8th day, GST levels in gills increased in 10, 25 and 50 $\mu\text{g/l}$ exposed groups while in digestive gland it increased in all the exposed groups (1, 10, 25 and 50 $\mu\text{g/l}$). Activities of GST in gills was positively correlated with Pb concentration, LPO and CAT whereas Pb concentration, LPO and SOD showed positive correlation in digestive gland (Table 3).

4. DISCUSSION

Under metal exposure, oyster accumulates metal at very high concentrations in different body parts to different extents (Marigomez et al., 2002) which generates several biological responses depending on metal concentration and duration of exposure (Hariharan et al., 2014). In the present experimental set up mortality of oysters were observed at the higher concentrations (10, 25 and 50 $\mu\text{g/l}$) of Pb exposed group. This could be due to increase in the levels of intracellular toxic metabolites. Moreover, accumulations of Pb in live oyster's gills was found to be approximately 2–4 times higher than in the digestive gland. The reasons for the higher concentrations in gills may be due to its continuous exposure to the surroundings and the large surface area of thin epithelium (Soldatov et al., 2007, Fernandez et al., 2010). Similar to these results, Prakash and Jagannatha Rao, (1995) also recorded high metal accumulation in gills than digestive gland of mussel *Perna viridis*. Alcutt and Pinto (1994) observed Pb equilibrium between tissue of clam, *Mercenaria mercenaria* and surrounding aquatic medium within short exposure (five days). The high accumulation of Pb in tissues of bivalves occurs due to the low excretion rates of amorphous granules and their immobilization in the shell (Viarengo, 1989).

In the current study, TBARS product was prominently increased in gills and digestive gland in all Pb exposed groups compared to unexposed (control) group. Significant and positive correlation ($p < 0.001$) was found between LPO and Pb concentration in both the tissues (Table 3). This infers that oxidative stress increases as a consequences of rise in ROS formation in lipid membranes due to gradual increase in Pb accumulation (Stohs and Bagchi, 1995; Ercal et al., 2001). It is known that marine organisms that are rich in polyunsaturated fatty acids increase ROS generation during metal exposure (Liu et al., 1997). This can lead to an imbalance in pro-oxidant and antioxidant processes, enhance oxidative damage and generate TBARS product (Manduzio et al., 2005). Another reason for high level of TBARS induction in both the tissues from exposed groups compared to control group

may be due to the structural and functional characteristics of both the organs. Firstly, It is important to point out that continuous oxidative load was experienced by gills in this study. As discussed earlier, an oyster's gill structure may be the reason that it showed more effect of Pb stress in successive concentrations (Soldatov et al., 2007). Secondly, since the digestive gland is the main organ involved in metabolic function, stress induced from various Pb concentrations may directly affect the metabolic pathway, resulting in both increased production and accumulation of ROS (Livingstone, 1992; Trombini et al., 2010). Consequences of these generated free radicals damages the membrane phospholipids and also change in fatty acid composition of molecules (Knowles and Donaldson, 1990; Hsu and Guo, 2002). This oxidative stress can further bring changes in the antioxidant enzyme activities (Manduzio et al., 2005, Gonzalez et al., 2015). The results of present study are corroborated by the findings of previous studies where researchers have found increased LPO in metal toxicity in mussels (Prakash and Jagannatha Rao, 1995; de Almeida et al., 2004; Vlahogianni and Valavanidis, 2007; Tsangaris et al., 2010) and prawn *Penaeus monodon* (Hariharan et al., 2012).

Superoxide dismutase is the first and crucial antioxidant enzyme which removes the superoxide radical through the process of dismutation to oxygen and hydrogen peroxide ($2O_2^{\bullet-} + H^+ \rightarrow H_2O_2 + O_2$) (Fridovich, 1975). It prevents generation of highly toxic $\cdot OH$ radicals. In the present study, SOD activity was slightly more in the digestive gland than gills. Moreover, positive correlation ($p < 0.001$) was found between the SOD and LPO as well as Pb concentration in both the tissues (Table 3). The observed positive correlation indicates that response of SOD was linked to Pb accumulation and its generated oxidative stress. SOD increased noticeably until the 6th day of exposure and decreased slightly on the 8th day in both the organs (prominently in gills). The initial increase in the SOD suggests the protective behavior of the cell against $O_2^{\bullet-}$ radicals produced by activation of the SOD initially while its depletion later on indicates the probable SOD degradation by ROS or its over utilization to overcome with Pb toxicity (Dafre et al., 2004). Furthermore, SOD activities in gills dropped more rapidly than those of the digestive gland suggesting that the detoxification function of digestive gland is more effective than the gills to counteract the high LPO level generated under plausible Pb toxicity (Trombini et al., 2010). This results are in the accordance with previous studies carried out by Jing et al. (2007) on oyster *Pinctada fucata* where SOD was decreased gradually in later part of Pb exposure experiment. Dai et al. (2012) were also recorded the inhibition of SOD in tilapia (*Oreochromis niloticus*) during dietary Pb exposure.

Catalase is a ubiquitous oxidoreductase antioxidant enzyme that breaks down the hydrogen peroxide molecule to water and oxygen molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$) and thus removes the toxicity of

H₂O₂ (Livingstone, 2001). In the present study, significant positive correlation ($p < 0.01$) was detected between CAT and Pb concentrations, LPO, SOD which suggest that SOD and CAT function together in a coordinated manner and neutralizes the generation H₂O₂, against the oxidative stress induced by Pb (Jena et al., 2009). Moreover, like SOD, CAT levels in both the tissues (gills and digestive gland) increased until the 4th and 6th day and declined slightly in the later phase of the experiment. These reductions in CAT impairs scavenging of H₂O₂ radical leading to the enhancement of LPO in both the tissues (Ercal et al. 2001). Similar results were obtained in green mussel *Perna viridis* under Pb exposure (Hariharan et al., 2014). According to our results, low activities of SOD and CAT indicate a non-significant contribution of these enzymes to antioxidant defense processes in the later phase of the experiment, which further may be associated with difficulty in compensating oxidative stress. Similarly, scientists have observed that CAT and SOD target Pb by forming the complexes with their substrates which is responsible for inhibition of these enzymes and deactivating them (Ercal et al., 2001; Marques et al., 2016). This decrease in SOD and CAT may results in increase in O₂⁻ radicals accumulation with combination of H₂O₂ generate hydroxyl [•]OH radicals (under classic Haber–Weiss reaction) resulting in rise of TBARS substances (Verlecar et al., 2008).

Glutathione-s-transferase is a major phase II-metabolic enzyme which plays a major role in detoxification of both xenobiotics and oxidative metabolic by-products (Van der Oost et al., 2003; Jozefczak et al., 2012). In the present study, gills and digestive gland showed initially less GST response which was below control level but it increased progressively from the 6th day onwards, indicating activation of detoxification mechanism in the gills as well as digestive gland cells. Apparently, the GST did not play an important role in neutralization of oxidative load initially, as judged by the low level of GST. Moreover, compensatory adaptive mechanisms between different biochemical biomarkers may occur (Regoli and Principato, 1995). Significant increase in GST activities in the later phase (8th day) of the experiment demonstrates the protective action of GST against reactive oxygen radicals to reduce Pb toxicity. This is also supported by the Pearson's correlation results where GST is positively correlated with the Pb concentration ($p < 0.001$), LPO ($p < 0.01$). This increase in GST activities may be a compensatory adaptive mechanism to neutralize increased levels of ROS when SOD and CAT were depleted from the 4th day onwards (Fernandez et al., 2010). Furthermore, digestive gland showed more GST activities as compared to gills. This elucidates that the gills are weak in detoxification process in the oxidative defense system as mentioned earlier. These results also corroborate the findings of Yan et al. (1997) where the authors found an effective increase in the glutathione content in the gills and digestive gland of green mussel *Perna viridis* after ~21 days of exposure to Pb stress. Similarly, Dafre et al. (2004) observed an

increase in glutathione level against the Pb stress in brown mussel *Perna perna*. In contrast, some researchers have found that Pb inactivates glutathione by binding it to sulfhydryl group present in it, which further depresses the glutathione level (Ahamed and Siddiqui, 2007). Thus, measurement of a single antioxidant is not enough to state a complete antioxidant defense under laboratory as well as field conditions. On the whole, our study shows that Pb generate the oxidative stress in gills and digestive gland and induces activation of SOD and CAT in the initial phase, whereas GST takes a position as a compensatory of the defense system in later stages. In this experiment, Pb toxicity arises may be due to two different, although related pathways: first, the ROS formation which includes $O_2^{\cdot-}$, H_2O_2 and $\cdot HO$ and second, the depletion of antioxidant reserves (Flora et al., 2002).

5. Conclusion

Based on the results of the investigated biochemical biomarkers LPO, SOD, CAT and GST we conclude that oxidative stress generated in gills and digestive gland of *C. madrasensis* is specifically depend on the concentrations and duration of Pb exposure. Furthermore, acute to high levels (≥ 10 $\mu g/l$) of dissolved Pb in surrounding seawater could be harmful to the physiology of the oyster. However, in future, similar studies should be conducted for longer exposure periods and with other biomarkers to present a complete overview of mechanisms of oxidative stress regulation in this sentinel species.

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Table 1 Results of three-way ANOVA on the Pb concentrations in gills and digestive glands of *C. madrasensis* under control and Pb (NO₃)₂ treated conditions (1µg/l, 10µg/l, 25µg/l, 50 µg/l) during 8 days experiment.

Variables	SS	<i>df</i>	MS	F	<i>p</i> value
Day	1.26266	3	0.42089	56.256	<i>0.000</i>
Concentration	4.82320	4	1.20580	161.167	<i>0.000</i>
Tissue	1.57094	1	1.57094	209.972	<i>0.000</i>
Days × Concentration	1.01723	12	0.08477	11.330	<i>0.000</i>
Days × Tissue	0.11394	3	0.03798	5.076	<i>0.003</i>
Concentration × Tissue	0.17656	4	0.04414	5.900	<i>0.000</i>
Days × Concentration × Tissue	0.16915	12	0.01410	1.884	<i>0.049</i>

df: degrees of freedom, SS: sum of squares, MS: mean sum of squares. *Italic* values denotes the significant level at $p < 0.05$

Table 2 Results of **three-way ANOVA** on the biomarker responses measured in gills and digestive gland of *C. madrasensis* under control and Pb (NO₃)₂ (1µg/l, 10µg/l, 25µg/l, 50 µg/l) treated conditions during experimental period.

		SS	df	MS	F	p value
LPO	Day	0.41706	3	0.13902	1237.8	0.000
	Concentration	1.54713	4	0.38678	3443.8	0.000
	Tissue	11.71074	1	11.71074	104270.2	0.000
	Day × concentration	0.08256	12	0.00688	61.3	0.000
	Day × tissue	0.01834	3	0.00611	54.4	0.000
	Concentration × tissue	0.37175	4	0.09294	827.5	0.000
	Day × concentration × tissue	0.08289	12	0.00691	61.5	0.000
SOD	Day	63.72	3	21.24	13.016	0.000
	Concentration	887.94	4	221.99	136.046	0.000
	Tissue	11166.56	1	11166.56	6843.508	0.000
	Day × concentration	73.51	12	6.13	3.754	0.000
	Day × tissue	61.19	3	20.40	12.500	0.000
	Concentration × tissue	853.18	4	213.30	130.720	0.000
	Day × concentration × tissue	72.99	12	6.08	3.727	0.000
CAT	Day	2724	3	908	111.7	0.000
	Concentration	3330	4	832	102.4	0.000
	Tissue	88	1	88	10.8	0.002
	Day × concentration	4662	12	389	47.8	0.000

	Day × tissue	128	3	43	5.3	<i>0.002</i>
	Concentration × tissue	394	4	98	12.1	<i>0.000</i>
	Day × concentration × tissue	2222	12	185	22.8	<i>0.000</i>
GST	Day	0.6502	3	0.2167	106.1	<i>0.000</i>
	Concentration	0.4196	4	0.1049	51.4	<i>0.000</i>
	Tissue	0.1014	1	0.1014	49.7	<i>0.000</i>
	Day × concentration	0.6426	12	0.0535	26.2	<i>0.000</i>
	Day × tissue	0.0513	3	0.0171	8.4	<i>0.000</i>
	Concentration × tissue	0.6018	4	0.1504	73.6	<i>0.000</i>
	Day × concentration × tissue	1.0891	12	0.0908	44.4	<i>0.000</i>

df: degrees of freedom, SS: sum of squares, MS: mean sum of squares. *Italic* values denotes the significant level at $p < 0.05$.

Table 3 Correlation (Pearson) between Pb accumulation and various antioxidant parameters in gills and digestive gland from control and exposed *C. madrasensis*.

	Gills					Digestive gland				
	Pb content	LPO	SOD	CAT	GST	Pb content	LPO	SOD	CAT	GST
Pb content	1.00					1.00				
LPO	0.91^c	1.00				0.91^c	1.00			
SOD	0.71^c	0.72^c	1.00			0.81^c	0.76^c	1.00		
CAT	0.59^b	0.58^b	0.53^a	1.00		0.67^b	0.68^b	0.61^b	1.00	
GST	0.77^c	0.68^b	0.36	0.47^a	1.00	0.79^c	0.70^b	0.53^a	0.42	1.00

Significance level at p<0.05 = a, p<0.01 = b, p<0.001 = c

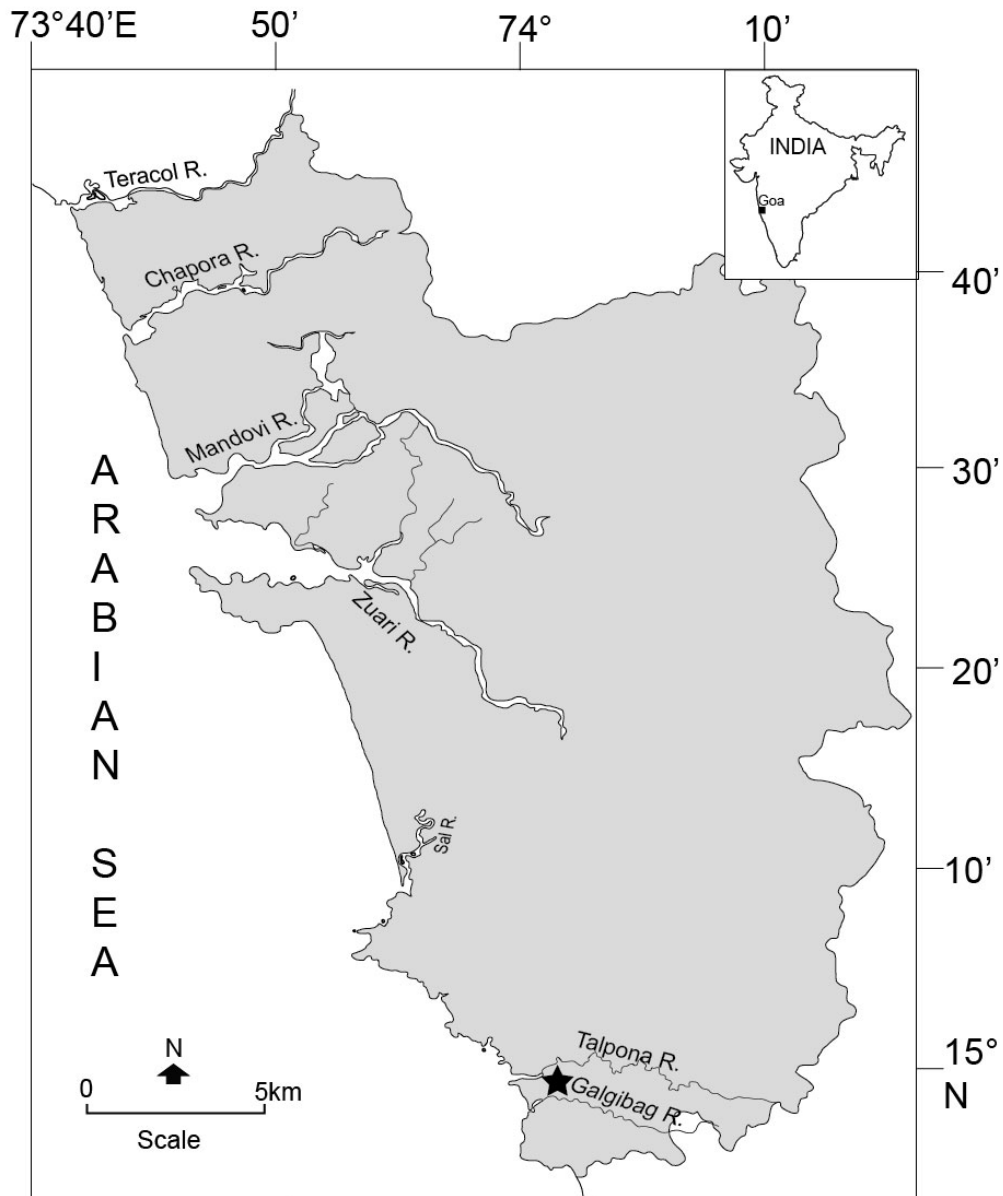


Fig. 1. The location of sampling site (★) at Galgibag estuary from Goa coast.

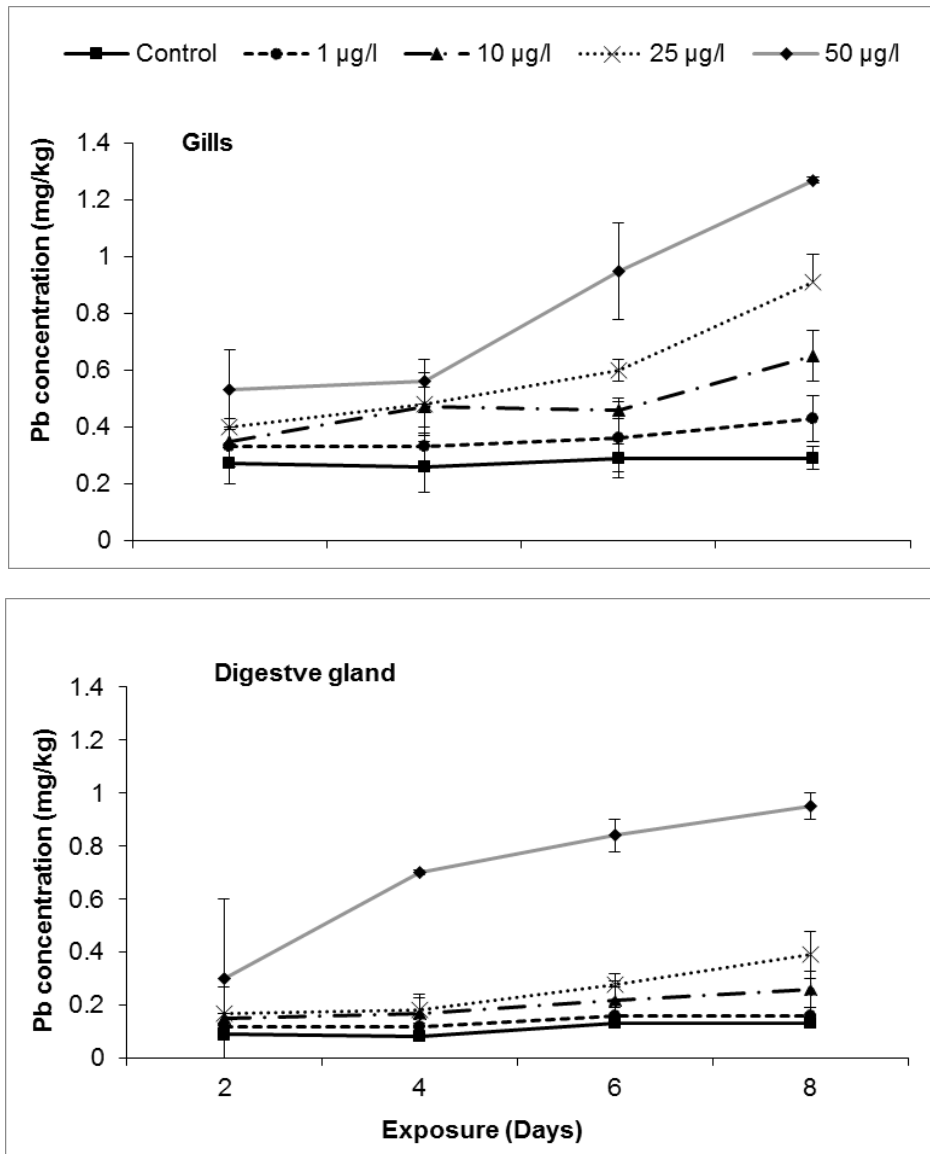


Fig. 2. Total average Pb concentrations (mg/kg) \pm SD (n = 3) in gills and digestive gland of control and exposed *C. madrasensis* to various concentrations of Pb (NO₃)₂ for 8 days.

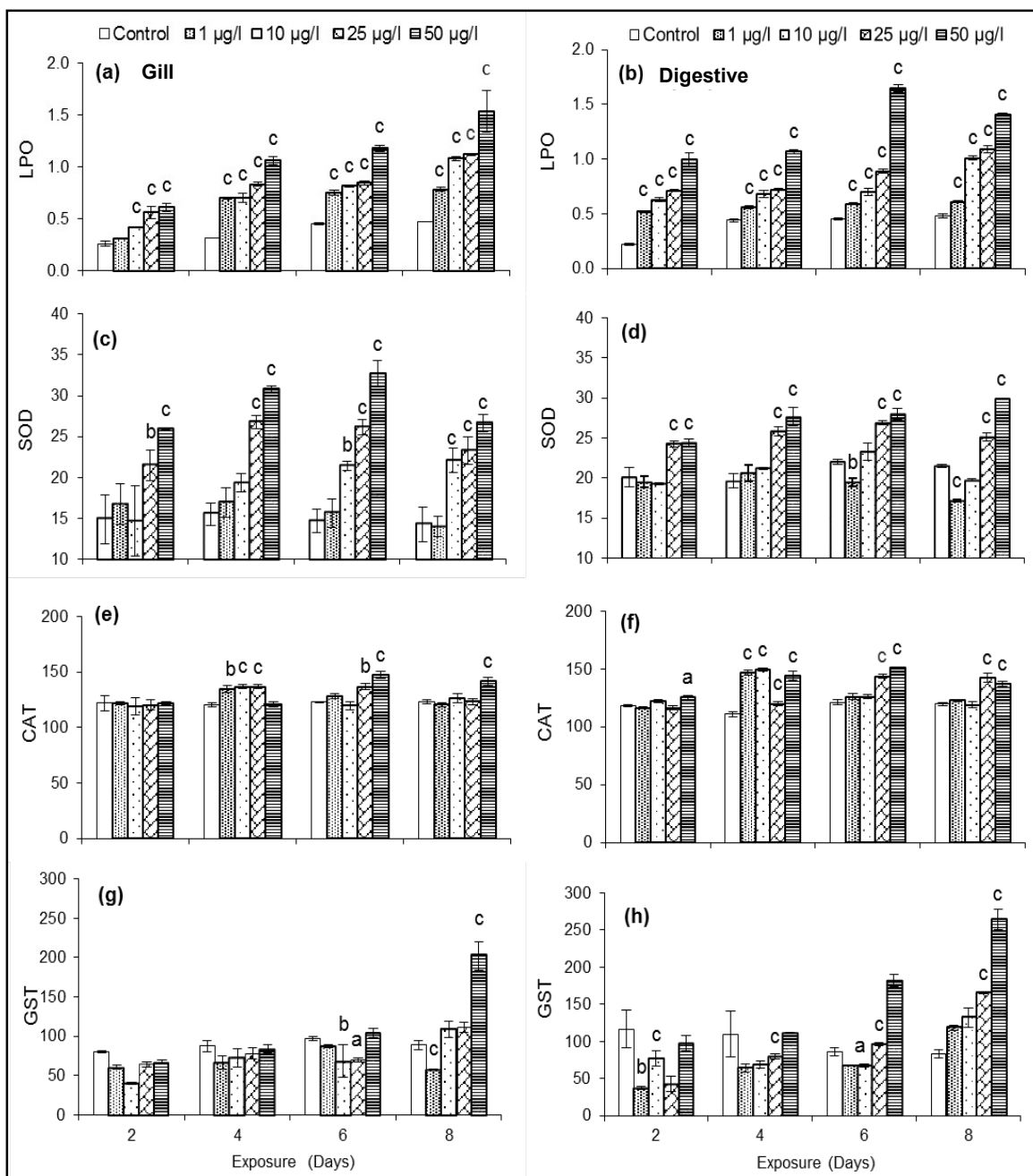


Fig. 3. Effect of Pb on (a and b) lipid peroxidation (nmol TBARS formed/mg protein), (c and d) superoxide dismutase (Unit/mg protein), (e and f) catalase (nkat/mg protein), (g and h) glutathione-s-transferase (nmol CDNB conjugate formed/min/mg protein) in gills and digestive gland of *C. madrasensis*. Data are expressed as average \pm SD (n = 3). Significant differences between treated group and control group denotes with superscript letter a, b and c at p < 0.05, p < 0.01 and p < 0.001 level respectively.