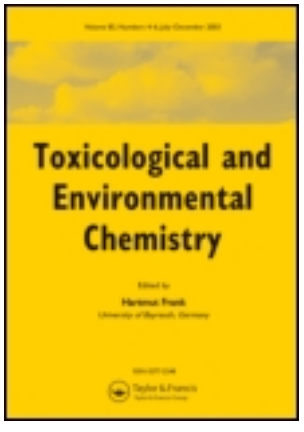

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Acute and chronic toxic effect of lead (Pb) and zinc (Zn) on biomarker response in post larvae of *Penaeus monodon* (Fabricus, 1798)

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Metal pollution produces damage to marine organisms at the cellular level possibly leading to ecological imbalance. The present investigation focused on the acute and chronic toxicity of lead (Pb) and zinc (Zn) by examining the effects of biomarker enzymes in post-larvae of *Penaeus monodon* (Tiger prawn). Antioxidant biomarker responses such as lipid peroxidation (LPO) and catalase (CAT) activity for Pb and Zn were determined following chronic exposure. Acute Lethal Concentration $_{50}$ (LC_{50}) values observed in the study at 96 h for Pb and Zn at $5.77 \pm 0.32\,mg\,L^{-1}$ and $3.02 \pm 0.82\,mg\,L^{-1}$, respectively. The estimated No Observed Effect Concentration and Lowest Observed Effect Concentration values for Pb were 0.014 and $0.029\,mg\,L^{-1}$ and that recorded for Zn was 0.011 and $0.022\,mg\,L^{-1}$, respectively. Among the two metals studied, toxicity of Zn was found to be greater to *P. monodon* than Pb. The activities of antioxidant defense enzymes and total protein content differed significantly from control following exposure to both metals. Overall, the biomarker studies demonstrated that alterations in antioxidant enzymes and induction of LPO reflect the consequences of heavy metal exposure in *P. monodon*.

Keywords: bioaccumulation; catalase; lipid peroxidation; lead; *Penaeus monodon*; toxicity; zinc

Introduction

Rapid growth in population, development of small and large-scale industries, expansion of harbor and tourism related activities, disposal of municipal and industrial wastes, and many other commercial activities have degraded the quality of coastal water causing serious health hazards to marine organisms and humans. Aqua culturists draw water either from rivers, estuaries or coastal areas and discharge their wastes back to these water bodies leading to adverse health hazards to marine organisms and fish consumers (Mubiana et al. 2005). It is well-established that heavy metals in estuarine and coastal ecosystems released from anthropogenic activities pose a threat to the marine environment. Previous studies in estuarine communities indicated that the quantity and species diversity of aquatic organisms are adversely affected by heavy metals (Padmini and Geetha 2007; Laxmi Priya et al. 2011).

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These heavy metals exist in various oxidation states that are reactive and toxic to marine animals even at low concentrations. Metals such as manganese (Mn), copper (Cu), iron (Fe), and zinc (Zn) act as micronutrients and play an essential role for biological functions of several proteins and enzymes. Among the essential trace elements, zinc (Zn) is known to be one of the most toxic metals to many organisms above certain concentrations and exposure duration (Maity et al. 2008). Metals such as mercury (Hg), cadmium (Cd), lead (Pb), and arsenic (As) are not essential in biological systems. Among the heavy metals, Pb produces severe toxicity to aquatic organisms even when present in lower concentrations. In Chennai coastal areas, heavy metals were found in high concentrations both in water, sediment and biota (Jayaprakash et al. 2005; Shanmugam et al. 2007; Seshan, Usha, and Deepthi 2012) and therefore, it is essential to study effects of heavy metals on metabolic pathways and their affinity to tissues, especially in crustaceans. Among the crustaceans, the tiger prawns are considered to be the indicators of marine and estuarine pollution, since they are widely distributed along the east coast of India. The tiger prawns *P. monodon* are an important commercial crustacean for the capture and culture fishing industries in India (Emerson Kagoo and Rajalakshmi 2002) and thus provide a suitable probe for biomarker studies.

Biomarker studies are useful to estimate the interaction between organisms and environmental contaminants (Jemac et al. 2009) in which majority of toxicity tests are conducted in laboratory scale to evaluate the toxic response of a biomarker, either concentration of the chemical or the response time. The mechanisms underlying toxicity of heavy metals involve the inhibition of the reactive oxygen species (ROS) generation. The overproduction of ROS such as H_2O_2 and superoxide radical (O_2^-) produces oxidative stress and affects various cellular processes and functioning of membrane systems and cell death (Radic et al. 2009). The toxic effects of ROS may be counteracted by antioxidant enzymes such as catalase (CAT). Therefore, the changes of enzymatic activities may indirectly indicate the adverse effects of heavy metals on living organisms. Thus, the present study was focused on accumulation of Pb and Zn and calculation of the No Observed Effective Concentration (NOEC), Lowest Observed Effective Concentration (LOEC) on post larval stage of *P. monodon*.

Materials and methods

Animal collection and acclimatization

Post larvae of *P. monodon* (PL 11-14) were procured from a prawn hatchery, located at Mamallapuram, Chennai, India. Larvae were immediately transported to the lab in air filled plastic bags and acclimatized in the aquaria glass tank with continuous aeration of filtered seawater for 7 days. The shrimps were fed with Higashimaru Prawn Feed (No. 3) during acclimatization. The animals were starved for 24 h prior to the initiation of the experiment. The physicochemical parameters of seawater in the experimental system were regularly monitored using standard methodologies (Grasshoff, Kremling, and Ehrhardt 1999).

Acute and chronic toxicity bioassay tests

Main stock solution of 0.1% (i.e., 1000 ppm) of Pb and Zn were prepared separately by dissolving 1.83 g lead (II) acetate or 2.08 g zinc chloride (MERCK, Germany) in 1 L ultrapure deionized water (Millipore-Milli-Q), respectively. The test concentrations for the

acute toxicity were selected from the range finding tests. The organisms were exposed in the test concentrations 2.25, 3.38, 5.1, 7.7, or 11.55 mg L^{-1} with control for Pb and 1.5, 2.25, 3.38, 5.1, or 7.7 mg L^{-1} with control for Zn.

The chronic toxicity test was assessed in 30 days exposure and in accordance with the USEPA (2002) requirements for life-stage chronic toxicity tests for fish was conducted as described by Stephan et al. (1985). Chronic concentrations were selected from the 2-week range finding test with Control, 0.014, 0.029, 0.058, 0.116, or 0.232 mg L^{-1} for Pb and Control, 0.012, 0.024, 0.048, 0.096, or 0.192 mg L^{-1} for Zn. The bioassay tests from the acute and chronic toxicity tests were conducted under continuous flow through methods described by USEPA (2002) and (Sprague 1973). Concentrations of Pb and Zn was measured at 24 h interval for acute toxicity tests and 10 days intervals for chronic toxicity tests. Dissolved Pb and Zn in seawater and toxicant medium were extracted and analyzed using Atomic Absorption Spectrometry (AAS – PerkinElmer AA analyst 800) as described by Grasshoff, Kremling, and Ehrhardt (1999). During the acute and the chronic toxicity tests, physicochemical variables including salinity, pH, water temperature and dissolved oxygen (DO) of the test chamber were analyzed twice a day by using pre-calibrated TKK-DOA water quality-monitoring probe WQC–24. During the chronic toxicity test, organisms were fed twice a day, and the unfed feed was removed. Definitive acute and chronic toxicity tests were conducted for continuous flow through method by using the Ismatec peristaltic pump.

Biomarker enzyme analysis

Biomarker enzyme analysis was carried out in whole-body tissue of *P. monodon* and samples were taken from test chamber termination following chronic exposure. Tissue was homogenized in a buffer containing sucrose −0.25 mol L^{-1}, 10 mmol L^{-1} – Tris and 1 m mol L^{-1} – EDTA adjusted to pH 7.4. The homogenates were then centrifuged at 3500 *g* for 15 min at −4°C. The resulting supernatant was used for the estimation of various biochemical assays. Total proteins were determined (Lowry et al. 1951). Lipid peroxidation (LPO) was estimated as described by Ohkawa (1979) expressed as *nmol* malondialdehyde (MDA) formed/mg protein. CAT was measured as the decay of hydrogen peroxide levels at 240 nm (Beers and Seizer 1952) and expressed as μmol of hydrogen peroxide consumed min^{-1} mg^{-1} per protein.

Bioaccumulation study

At the end of the chronic exposure, survived test organisms were removed, tissue washed with distilled water, and oven dried at 95°C. The tissue was ground to fine powder and digested (APHA, AWWA, and WPCF 1998), and metal accumulation determined using atomic absorption spectrophotometer (AAS - PerkinElmer AA Analyst 800).

Statistical analysis

Results from both acute and chronic toxicity test were calculated based on the dissolved concentrations of Pb and Zn. The 96 h LC_{50} and 95% confidence limits were calculated using Probit Analysis based on the mortality of the test organisms at each dissolved concentration (Finney 1971). In the chronic toxicity test, NOEC and LOEC values were calculated based on the survival of the test organisms on the 30th day of the chronic toxicity

Table 1. Nominal and measured Pb and Zn concentrations in acute and chronic toxicity test solutions and relative percentage of recovery.

Toxicity test	Exposed metals	Nominal concentration in test chamber ($mg\,L^{-1}$)	Measured concentration in test chamber ($mg\,L^{-1}$)[a]	% of recovery Nominal *vs.* measured concentration[b]
Acute exposure	Pb	2.25	2.11 ± 0.06	93.8
		3.38	3.20 ± 0.08	94.7
		5.1	4.88 ± 0.20	95.7
		7.7	7.37 ± 0.25	95.7
		11.55	10.74 ± 0.34	93.0
	Zn	1.5	1.36 ± 0.04	90.7
		2.25	2.08 ± 0.01	92.4
		3.38	3.18 ± 0.05	94.1
		5.1	4.67 ± 0.35	91.6
		7.7	6.83 ± 0.27	88.7
Chronic exposure	Pb	0.014	0.014 ± 0.004	100.0
		0.029	0.029 ± 0.008	100.0
		0.058	0.056 ± 0.011	96.9
		0.116	0.108 ± 0.010	93.4
		0.232	0.230 ± 0.014	99.2
	Zn	0.012	0.011 ± 0.004	94.4
		0.024	0.022 ± 0.010	93.3
		0.048	0.044 ± 0.011	92.6
		0.096	0.084 ± 0.007	88.2
		0.192	0.184 ± 0.012	96.0

Notes: [a]Measured values are means from samples collected every 24 h (24, 48, 72, and 96 h) in acute tests and 10 days (Initial day, 10th day, 20th day, and 30th day) ones in chronic tests.
[b]Relative percentage of recovery between Nominal *vs.* Measured concentrations.

test (Dunnett 1955). Enzymes values were expressed as mean ± SD and analyzed by Graphpad Prism 5 software. One-way ANOVA in conjunction with Dunnett's test was used to determine if the treatments were significantly different from the control group ($p < 0.05$).

Results

Water quality and concentration of Pb and Zn in toxicant medium

Lab seawater quality parameters were consistent throughout the tests, ranging as follows: temperature: 25.4 ± 0.7°C; DO: $6.3 \pm 0.6\,mg\,L^{-1}$; pH: 7.1 ± 0.5; salinity: 27.7 ± 0.5; Pb: $1.7\,\mu g\,L^{-1}$ and Zn: $3.2\,\mu g\,L^{-1}$. For all bioassays, the nominal and measured concentrations of toxicant were compared to evaluate the stability of exposed concentration in test chamber and determine the accuracy of the analytical method. The mean dissolved concentration and % of recovery between the results of nominal and measured concentrations are summarized in Table 1.

Acute toxicity test

The LC_{50} values and their 95% confidence limits are presented in Table 2 based on the measured concentration. The 96 h LC_{50} of Pb and Zn at different concentrations were

Table 2. Lethal concentrations (LC_{50}) of Pb and Zn depending on exposure time (24–96 h) for *P. monodon* and 95% confident intervals.

	Time of exposed	LC_{50} (mg L^{-1})	95% confident intervals (mg L^{-1})	
			Lower	Upper
Pb	24	29.23 ± 16.88	16.42	326.36
	48	13.00 ± 1.52	10.26	20.53
	72	8.85 ± 0.87	7.42	11.47
	96	5.77 ± 0.38	4.96	6.76
Zn	24	10.17 ± 0.96	7.87	18.16
	48	8.55 ± 0.80	6.51	14.79
	72	5.49 ± 0.21	4.53	7.30
	96	3.58 ± 0.20	2.74	4.83

Table 3. Percentage of survival, LOEC, NOEC, and Chronic values of Pb and Zn exposed to *P. monodon*.

Toxicant used	Concentration (mg L^{-1})	% of Survival	NOEC (mg L^{-1})	LOEC (mg L^{-1})	Chronic value (mg L^{-1})
Pb	Control	100	0.014	0.029	0.020
	0.014	100			
	0.029	75			
	0.056	50			
	0.108	35			
	0.230	15			
Zn	Control	100	0.011	0.022	0.016
	0.011	100			
	0.022	70			
	0.044	55			
	0.084	50			
	0.184	30			

5.77 ± 0.32 and 3.02 ± 0.82 mg L^{-1}, respectively. The survival rate of control organisms in both Pb and Zn exposure was 100%. Mortality was absent from the initial time of exposure to different concentrations of Pb and Zn, but it was amplified with longer exposure duration and increased concentrations. For the three acute toxicity tests conducted for Pb and Zn, the average LC_{50} was found to be 24, 48, 72, and 96 h, respectively. Significant correlation was observed with increasing time and mortality rate and the r^2 values of Pb and Zn were 0.77 and 0.98, respectively.

Chronic toxicity

The survival % exposed organisms at the end of chronic exposure and results of NOEC, LOEC and chronic are given in Table 3. The chronic toxicity tests revealed that the survival of *P. monodon* decreased with increasing exposure concentrations (Table 3). The % survival for *P. monodon* in the first two concentrations of Pb (0.14 and 0.29 mg L^{-1}) and

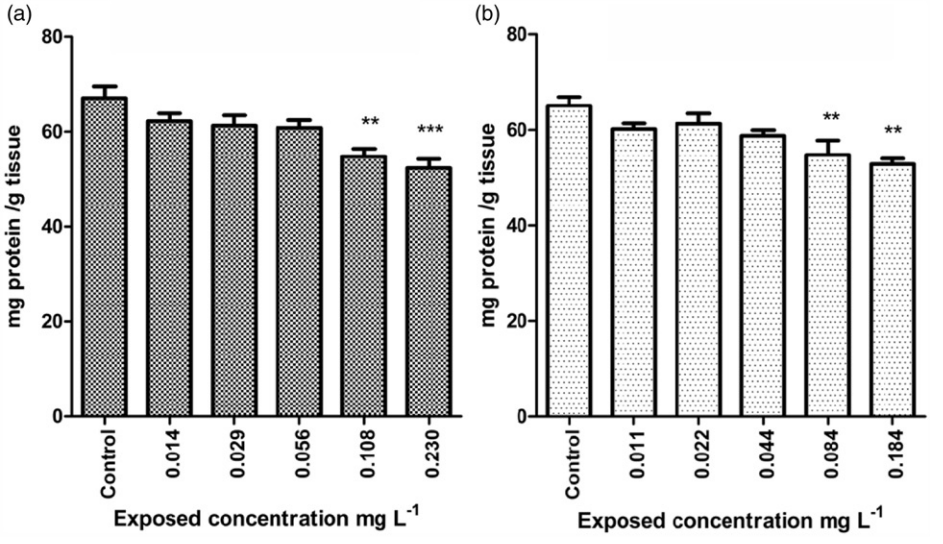

Figure 1. Variation of total protein level in *P. monodon* exposed to Pb and Zn in chronic toxicity test.
Notes: Total protein in the whole body of *P. monodon* after 30th day of exposure to Pb and Zn; values are mean ± standard deviations, $n = 2$, significantly different from the control value; $^{**}p \leq 0.001$; $^{***}p \leq 0.0001$.

Zn (0.01 and 0.02 mg L^{-1}) were not significantly different from control whereas significant difference was observed at other exposure concentrations for both Pb and Zn. Minimal survival of organisms was observed in the highest concentration of 0.23 mg L^{-1} Pb and 0.18 mg L^{-1} Zn (15% in Pb and 30% in Zn) after 30 days. The NOEC and LOEC values calculated based on survival of test organisms, were 0.014 and 0.029 mg L^{-1} for Pb and 0.01 and 0.02 mg L^{-1} for Zn, respectively.

Biomarker studies

Significant reduction of protein content was observed in whole-body tissue of *P. monodon* subjected to 30 days exposure compared with control. Maximal reduction in protein content (Figure 1) was observed at the highest concentrations of Pb (0.23 mg L^{-1}) and Zn (0.18 mg L^{-1}).

Oxidative damage in whole-body tissue of *P. monodon* exposed to Pb and Zn showed varying responses. No significant change in LPO level was observed at 0.014 and 0.029 mg L^{-1} Pb and 0.01 and 0.02 mg L^{-1} Zn compared to control. *P. monodon* exhibited a consistently increasing pattern of LPO levels with rising exposure concentrations. With both metals, maximal increase of LPO was observed at higher concentrations particularly after 30 days. Zn showed more induction in LPO level at 0.18 mg L^{-1} (Figure 2).

The activities of CAT in the lowest two concentrations of *P. monodon* of both metals were increased numerically. CAT activity was significantly elevated at higher concentrations in Pb and Zn exposed animals (Figure 3).

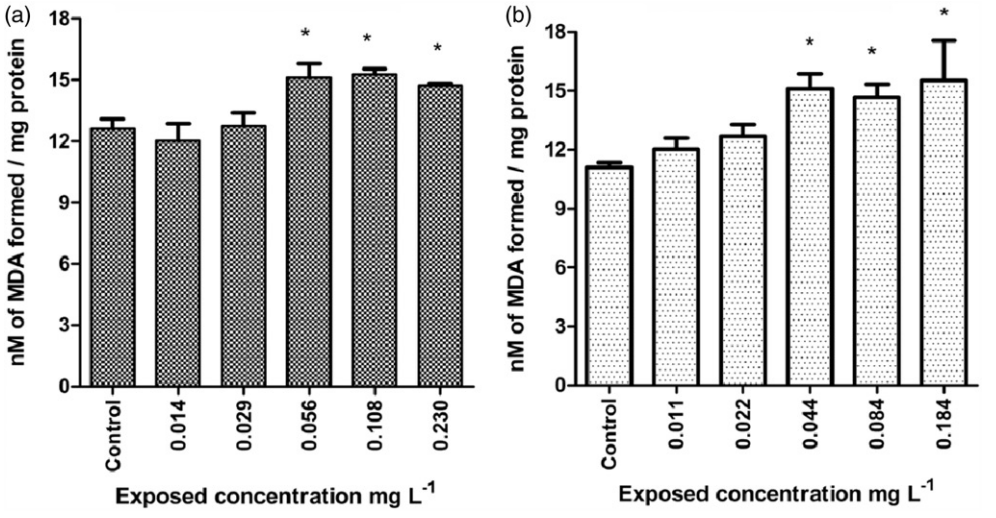

Figure 2. Variation of LPO in *P. monodon* exposed to Pb and Zn in chronic toxicity test.
Notes: LPO in the whole body of *P. monodon* after 30th day of exposure to Pb and Zn; values are mean ± standard deviations, $n = 2$, significantly different from the control value; $*p \leq 0.05$.

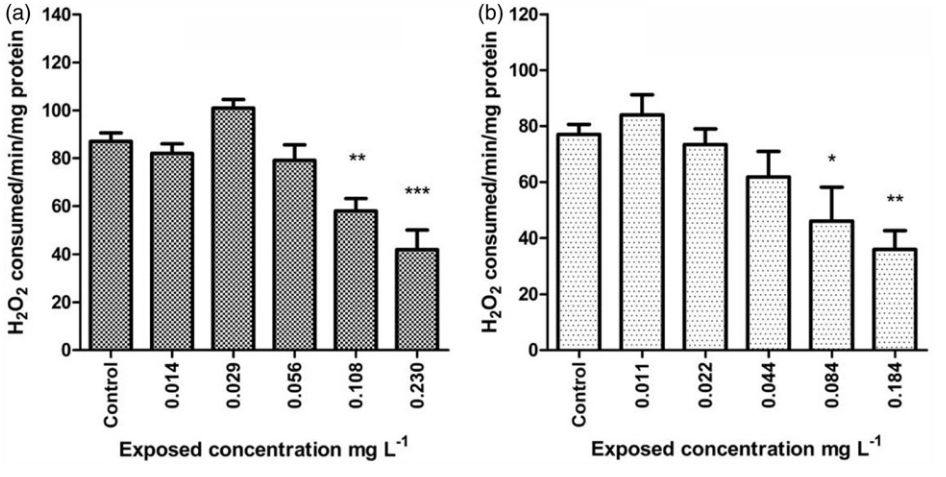

Figure 3. Variation of CAT in *P. monodon* exposed to Pb and Zn in chronic toxicity test.
Notes: CAT in the whole body of *P. monodon* after 30th day of exposure to Pb and Zn; values are mean ± standard deviations, $n = 2$, significantly different from the control value; $*p \leq 0.05$; $**p \leq 0.001$; $***p \leq 0.0001$.

Metal accumulation

P. monodon exposed to Pb and Zn shown in Figure 4 displayed significant increasing trend in concentration of metals in tissues. The results indicated increased accumulation of Pb and Zn with rising exposure concentration.

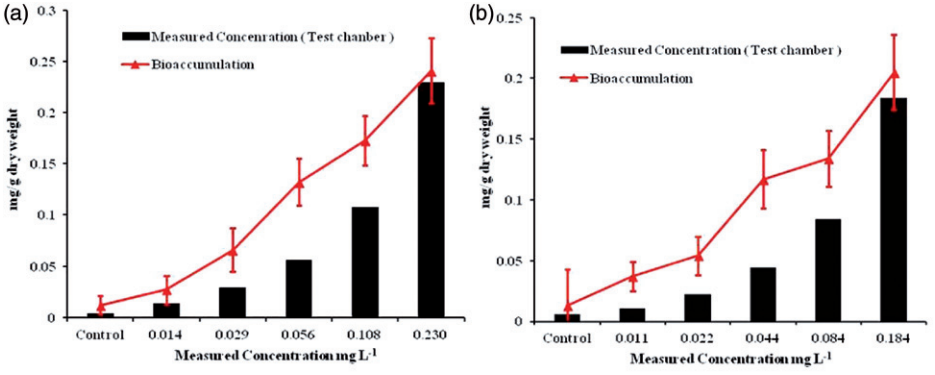

Figure 4. Bioaccumulation of Pb and Zn in PL of *P. monodon* on exposure to different concentrations.
Notes: Accumulation in the whole tissue of *P. monodon* after 30th day of exposure to Pb and Zn; values are mean ± standard deviations, $n = 2$.

Discussion

Acute toxicity

The acute toxicity tests clearly indicate that the post larvae of *P. monodon* were more sensitive to Zn than Pb. In the acute toxicity test, animal behavior is a key indicator for toxic effect. Behavioral responses of test animals were observed at different test concentrations with no adverse responses noted in control. Hyperactivities of animals were observed in initial stages of exposure to higher concentration and all concentrations at the end of the acute test, which may be associated with enzymatic and respiratory processes in aquatic animals (Shetty, Deepa, and Alwar 2007; Tawari, Igetei, and Okoidigun 2008). The acute toxicity of Pb to marine and freshwater invertebrates is generally less than that of Cu, Cd, Co, Hg, and Zn (Wang, Kong, and Wu 2007). In the present study, LC_{50} value in *P. monodon* was compared with toxic effects of heavy metals to marine and estuarine invertebrates as described by McLusky, Bryant, and Campbell (1986). The order of toxicity of metals is generally, Cd > Cu > Zn > Pb. The present study also observed a similar pattern, namely Zn > Pb. The results confirm that the essential metal Zn was toxic to organisms when exposed to higher concentrations as observed by Prazeres, Martins, and Bianchini (2011).

The 96 h LC_{50} Pb values observed in *P. monodon* were comparable with reported values of 7.28 mg L^{-1} by Fafioye and Ogunsanwo (2007) in *P. monodon* and 7.22 mg L^{-1} by Chinni, Ritindra, and Prabhakara (2002) in *Penaeus indicus*. The 96 h Zn LC_{50} values in *P. monodon* are 2.29 ± 0.86 is comparable to the study conducted by Barbieri (2008) with 3.31 mg L^{-1} for *Farfantepenaeus paulensis*.

Chronic toxicity

In chronic exposure, survival was found to be the more sensitive measure for metal toxicity than growth (Versteeg et al. 2006). Since data on chronic toxicity of *P. monodon* exposed to Pb and Zn is scarce, results obtained in the present study were compared with available date on allied crustacean species. The present observations and the results obtained viewed in the context of the ASEAN marine water quality criterion (AMWQC) of Pb and Zn was similar. The chronic values were calculated based on the geometric mean of NOEC and

LOEC, 0.02 and 0.02 mg L^{-1} for Pb and Zn, respectively, and chronic exposure survival results are in agreement with these levels for *P. monodon*. Previous studies on crustaceans (Fafioye and Ogunsanwo 2007) indicated that the chronic exposure of the metals produced physiological stress in crustaceans. In these conditions, enhanced production of antioxidant enzyme activity during chronic exposure was correlated with increased rate of mortality at higher concentrations as evident in the present chronic studies. The % animal survival markedly declined toward the end of the experiment (25–30 days) in this study for both Pb and Zn. The declined survival might be due to the long-term exposure of shrimps to the metals. This suggests that bioaccumulation of metals are generally higher at lower exposure concentrations (McGeer et al. 2003). The present observation of biomarker studies also substantiates higher mortality frequency at high concentration of Pb and Zn.

Biomarker studies

The reduction in the protein level in shrimps exposed to metals may be due to catabolism of proteins under stress and also to long term exposure to toxicants (Rajkumar et al. 2011). Antioxidant enzymes are commonly employed as biomarkers of oxidative stress. However, the response to pollution varies for different species, enzymes and single or mixed contaminants, and even greater variability was found in field situations (Livingstone 2001). The present study observed a varied response of antioxidants and protein content in whole-body tissues of *P. monodon* when exposed to Pb and Zn. The results showed that LPO was significantly higher in *P. monodon* treated with Pb and Zn, which indicates that oxidative stress. This might be due to increased production and accumulation of ROS. All marine organisms contain high levels of polyunsaturated fatty acids that are substrates for LPO (Liu et al. 1997). Increased LPO leads to production of MDA that enhances formation of free radicals from polyunsaturated fatty acids in cell membranes (Rameshthangam and Ramasamy 2006). The results of present study are in agreement with the studies increased LPO levels in marine animals exposed to contaminants (Tsangaris et al. 2010). Free radical scavenging enzymes such as CAT are the first line of defense against oxidative injury (Farombi, Adelowo, and Ajimoko 2007). In the present study, CAT response to toxic chemicals showed a bell-shaped trend with an initial increase in activity due to enzyme induction followed by a decrease in activity due to enhanced catabolic rate and/or direct inhibition by toxic chemicals (Viarengo et al. 2007). Such trends in CAT activities were found in mussels in polluted areas according to the levels and duration of pollutant exposure (Pampanin et al. 2005). A decreased CAT activity was observed in *P. monodon* exposed to higher concentration both Pb and Zn. Similarly, decreased CAT level was observed in mussels transplanted at polluted sites have been found in addition to a reduced capability of neutralizing ROS and an increased susceptibility to oxidative stress (Pampanin et al. 2005). Accordingly, decreased CAT activity in the present study may be associated with difficulty to compensate oxidative stress.

Overall, the enzyme response to Pb and Zn showed a bell-shaped activity of antioxidant defense mechanism. These types of activity indicate that Pb and Zn induced increased antioxidant enzymes at certain concentrations where high levels of concentration reduce the antioxidant defenses and reduction of antioxidant enzymes resulted in death of exposed organisms (Valavanidis et al. 2006). Results of chronic studies indicated that the rate of mortality increases with elevation in concentration and for a particular concentration with longer exposure time.

Bioaccumulation

Invertebrates, particularly crustaceans, were sensitive to metals. Chronic toxicity of *P. monodon* was associated with high levels of metal accumulation with increasing exposure concentration and increasing level is approximately double exposed concentration (Figure 4). In an overview of the bioaccumulation, the lower exposed concentration seems to accumulate more metals than the higher concentrations. Similar results were also noted by Chinni, Ritindra, and Prabhakara (2002) *P. indicus* during acute exposure to Pb. Ahsanullah and Ying (1995) found crustaceans accumulate metals more than the exposed concentration due to binding of metals to metallothionein proteins in the hepatopancreas.

Conclusion

Chronic exposure even at low concentration of Pb and Zn affects the metabolic activity in *P. monodon*. The LC_{50} and chronic values obtained from toxicity studies provided data on the effect of pollutants especially Pb and Zn are useful in screening potentially toxic substances. Data obtained from the study will be useful to determine the concentrations of single contaminants that may produce ecologically significant effects and data might also be used to establish acceptable environmental standards. The assessment of the acute and chronic toxicity is the primary step to determine the criterion maximum concentration and criterion continuous concentration on estuarine organisms, which may be evaluated in the future as similar studies have not yet been carried out for the Chennai coast. Based on the present study, it is concluded that the further research is needed to optimize the dietary constituents, feeding and growth rate for post larvae of *P. monodon* in chronic toxicity tests.

Acknowledgments

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