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Immunomodulation in the marine gastropod *Haliotis diversicolor* exposed to benzo(*a*)pyrene

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ABSTRACT

It has been reported that environmental pollutants in the aquatic ecosystem could weaken immune competence of organisms. The purpose of the present study was to investigate the effects of benzo(*a*)pyrene [B(*a*)P] on immunomodulation in marine gastropods and to see if these effects are caused by or related to the generation of reactive oxygen species. In our present study, the marine gastropod *Haliotis diversicolor* was exposed to sublethal concentrations (0.01, 0.02, 0.04 and 0.08 mg L^{-1}) of B(*a*)P for 7 d under laboratory conditions and the alterations of hematological parameters like haemocyte count, haemocyte viability, protein content and immune components like phenoloxidase, phagocytosis and superoxide anion generation were measured. In addition, the changes in lysozyme activity, antibacterial activity due to the effect of B(*a*)P on abalone were analysed. B(*a*)P was found to decrease significantly the total number of circulating haemocytes. Intracellular superoxide anion generation and phenoloxidase significantly increased on exposure to B(*a*)P, whereas phagocytic activity was decreased significantly at higher concentration. Significant alterations were found in the uptake of neutral red and the observed alterations of hematological parameters and immune components tested indicated the generation of immunotoxicological effects on abalone due to B(*a*)P exposure. The results demonstrate a possible relationship between B(a)P and the immunological parameters of abalone studied.

1. Introduction

The rapid increase in anthropogenic activities leads to environmental pollution during last few decades and more importantly, pollution of the aquatic ecosystem is alarming. Pollution in the marine environment may be one of the reasons for disease occurrence in marine organisms, due to adverse effects of xenobiotics on the physiology, especially the immune system of the animal. Human activities increased the level of contaminants in aquatic ecosystem especially man-made organic foreign compounds enter and are widespread in coastal regions by various routes, including direct discharge, direct use, land run-off, atmospheric deposition, abiotic and biotic movement, and in various food chain transfers (Livingstone et al., 1992). Several studies reported a possible relationship between various xenobiotics and the stress that it causes in animals, as well as diseases (Sinderman, 1993). The effects of a pollutant on animal immune system may be a direct action or an indirect one. It is noteworthy that alterations in the homeostatic mechanisms, including the immune system are critical, since these predispose an animal to severe infection and subsequent disease (Fournier et al., 2000).

Pipe et al. (1999) reported that environmental contaminant effects may result from direct toxic action on the tissues or from subtle alterations in homeostatic mechanisms, which reflects the degree of pollutant induced stress and give an early indication of disease susceptibility and, ultimately, survival of the organisms. Polycyclic aromatic hydrocarbons (PAHs) is a serious organic pollutant in marine environment which includes B(a)P in which the molecular structure includes two or more fused aromatic rings and adjacent rings share two or more carbon atoms.

Many chemicals entered into the marine environment due to industrial and agricultural activity have been implicated in ecotoxicological effects mediated through immunotoxic mechanisms in exposed organisms. The dependence of immune responses on receptor binding allows the system much susceptible to interference by pollutant which may activate the inappropriate responses in the organisms (Thiagarajan et al., 2006). In addition, any xenobiotic which interrupt the associated cellular metabolism and the production of ATP will decrease the immune cell viability and their functions (Buttgereit et al., 2000). Examination of the available information reveals that some species like bivalve molluscs (*Mytilus edulis*, *Crassostrea virginica* and *Perna viridis*) have received a great deal of attention on immunotoxicity due to xenobiotics (Sami et al., 1992, 1993; Coles et al., 1994, 1995; Pipe et al., 1999; Thiagarajan et al., 2006), while gastropod abalone have only been investigated in relation to ecological factor (Cheng et al., 2004a–e). This

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is clearly a deficiency since a distorted view of the effects of immunotoxic chemicals in biota may have arisen (Galloway and Depledge, 2001).

Molluscs have been extensively used in marine pollution monitoring programmes in recent years. The gastropods that have a wide geographical distribution in coastal waters are known to readily accumulate pollutant and show various physiological and biochemical responses providing information on the general status of contamination in the coastal environment and health of the animal itself. A variety of biomarkers has been used to monitor the level of environment pollution (Nicholson, 2003; Siu et al., 2004; Nicholson and Lam, 2005; Thiagarajan et al., 2006). Studies on immunomodulation in molluscs have shown drastic changes in animal's immune competence upon exposure to different xenobiotics (Pipe et al., 1999; Pavlica et al., 2000; Sauve et al., 2002; Gagnaire et al., 2004; Thiagarajan et al., 2006). The immune defence system of molluscs is comprised of cell-mediated and humoral mechanisms, in which the haemocytes play a key role (Cheng, 1981). Migration of haemocytes stimulated by antigenic challenge leads to phagocytosis and intracellular degradation of the pathogen by means of lytic enzymes (Pipe, 1990) or the production of highly reactive oxygen metabolites (Pipe, 1992) as cited in Pipe et al. (1999). Abalones are large algivorous marine gastropods, and one of the most important commercial species of gastropods in coastal aquaculture. Studies on abalone immune functions are very few, and most of them mainly focused on the relationship between ecological factors and the immune response of abalone. (Malham et al., 2003; Cheng et al., 2004a–c; Hooper et al., 2007). Until now to our knowledge there are no studies on the effects of organic pollutants concerning immune competence of the abalone. Thus, this investigation is the first to deal with immunomodulation in *Haliotis diversicolor* exposed to sublethal concentrations of organic pollutant.

The aim of the present study thus was to investigate the effects of B(*a*)P on the immune function of the gastropod *H. diversicolor*. B(*a*)P is considered in the present study because it is the one of the important organic pollutant for which there are sufficient toxicological evidence to allow the setting of a guideline. The parameters studied were related to the ability of the blood cells to destroy invading pathogens and included changes in the number of the circulating haemocytes, phenoloxidase enzyme activity, superoxide anion generation, phagocytosis, lysosomal membrane stability, lysozyme activity and antibacterial activity. A rapid assay was adopted with a view to monitoring the overall health status of the organisms under xenobiotic stress.

2. Materials and methods

2.1. Animals

Live healthy *H. diversicolor* (55 ± 5 mm in shell length) obtained from the Zhangpu abalone farm of Fujian Province were acclimatized to the laboratory conditions with temperature 24 ± 1 °C, salinity of 30 ± 1‰ and pH 7.8 ± 0.1 for seven days before experimentation. Animals were reared in 80 L PVC tanks containing 40 L seawater treated with sand filtration, kept on a natural daylight cycle and fed with the marine alga *Gracilaria tenuistipitata* during the acclimation and experimental period.

2.2. Chemicals

Benzo(*a*)pyrene (purity, 99%), 3,4-dihydroxy-L-phenylalanine (L-DOPA), *Laminarin digitata, Micrococcus lysodeikticus*, nitro blue tetrazolium (NBT) and LPS from *E. coli* were obtained from Sigma (Sigma Chemicals, St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.3. Benzo(a)Pyrene bioassay test

Acute toxicity (96 h) study was carried out to determine the lethal (LC_{100}), median lethal (LC_{50}) and sublethal (LC_0) level of B(*a*)P to *H. diversicolor* by static renewal method (EPA/ROC, 1998). Stock solution of B(*a*)P was prepared at 1 part per thousand (PPT) using acetone (HPLC grade). From this stock solution, the following concentrations 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg L^{-1} were prepared. Ten abalones were introduced into each concentration, in 10 L glass aquaria and the seawater and test chemical renewed every day. No feed was provided during the 96 h bioassay test period. Any dead abalone was removed immediately from the test medium. Duplicates were performed for each concentration. The percentage mortality of abalones was noted after 96 h and percent mortality was analysed using probit regression to find out the 96 h LC_{50} value as described by Finney (1971).

2.4. Toxicity test for immunotoxicological biomarkers

To assess the changes in biomarkers, abalones were divided into six groups of 10 specimens each. Group I abalones were reared in normal seawater. Group II were used as solvent control (acetone); and abalones in group III to VI were exposed to seawater containing 0.01, 0.02, 0.04 and 0.08 mg L^{-1} B(*a*)P, respectively, which were the sublethal concentrations at which 0% mortality occurred by 96 h. Glass aquaria (30" × 25" × 20") were used in the experiments. The test solution and seawater was renewed daily and the abalones were fed with *G. tenuistipitata* during the experimental period. After 7 d of exposure the experiment was terminated and the animals were sacrificed to assess the immunotoxic parameters.

2.5. Assessment of immunotoxicological biomarkers

2.5.1. Isolation of plasma and haemocytes

Haemolymph was collected by cutting the blood sinus in the adductor muscle and was immediately placed on ice to retard cell clumping. About 1.5 mL of haemolymph collected from each abalone was immediately used for determining cellular immune parameters. The rest of the haemolymph was centrifuged at 3700 rpm for 15 min at 4 °C to collect plasma, which was then stored at −70 °C for subsequent analysis. The plasma (=supernatant) was aliquoted separately and used for PO assay and lysozyme activity. The haemocyte pellet was resuspended in equal volume of phosphate buffer saline (0.1 M, pH 7.4) and the haemocytes thus prepared were used for super oxide assay. Haemolymph from individual abalone was examined and plasma and haemocytes thus isolated were not pooled for immunoassays.

2.5.2. Total haemocyte count

Total haemocytes in haemolymph was determined according to the method of Barracco et al. (1999). A sample of 50 μL of haemolymph was added to a haemocytometer and counted in microscope under 40 × magnifications.

2.5.3. Phenoloxidase assay

Plasma phenoloxidase activity in *H. diversicolor* was assessed following the procedure of Asokan et al. (1997). Briefly, 100 μL of plasma, from control or experimental groups were mixed with 100 μL of PBS and incubated for 20 min at 22 °C. At the end of incubation, 2 mL of 1 mg mL^{-1} L-DOPA was added and further incubated for 5 min at 22 °C. All incubation experiments were performed in the dark. After incubation, the O.D. of the samples were read at 460 nm in a Ultrospec 2100 pro UV/visible

spectrophotometer against a reagent blank containing 200 μL buffer and 2 mL L-DOPA. Plasma samples were estimated for protein content by the method of Bradford (1976). To check that the observed oxidation of L-DOPA by plasma of *H. diversicolor* was due to PO but not peroxidase, PO assays were performed in the presence of thiourea (a known inhibitor of PO (Prabhakaran et al., 1969). In this experiment, 200 μL of plasma was preincubated with 100 μL of 5 mM thiourea for 20 min at 22 °C. An aliquot of 200 μL of this reaction mixture was incubated with 2 mL of 1 mg mL^{-1} L-DOPA for 5 min at 22 °C. For the controls, PBS buffer was substituted for thiourea. The optical density of both control and experimental was measured at 460 nm. The phenoloxidase activity is expressed in units of mg $protein^{-1}$ min^{-1}.

2.5.4. Total prophenoloxidase (proPO)

Dose response to proPO activation was carried out using laminarin and LPS. Briefly, samples of 200 μL plasma was preincubated with 100 μL of laminarin at various concentrations (0.1–10 mg mL^{-1}) for 20 min at 22 °C before L-DOPA was added and PO activity assayed. Under identical experimental conditions, activation of proPO in plasma by LPS was tested at concentrations ranging from 0.01 to 0.5 mg mL^{-1}. Samples of 200 μL plasma of control and B(*a*)P exposed abalone was preincubated with 100 μL of laminarin(1 mg mL^{-1}) or LPS (0.05 mg mL^{-1}) for 20 min at 22 °C before L-DOPA was added. A known volume (200 μL) from each reaction mixture was allowed to react with L-DOPA and PO activity was assayed. The amount of measurable proPO for each concentration was determined by subtracting the PO activity in buffer-control from that of positive control.

2.5.5. Superoxide anion generation assay

The *in vitro* generation of superoxide anion (O_2^-) by the haemocytes of abalone was assessed by NBT following the procedure of Arumugam et al. (2000). Breifly, haemocyte suspension obtained from individual abalone was divided into three 250-μL aliquots and one aliquot was used as control, while to the other 50 μL of 2 mg mL^{-1} laminarin or 50 μL of 0.1 mg mL^{-1} LPS was added, as a stimulant for O_2^- generation (optimal concentration of laminarin and LPS was determined by using varying concentration, data not shown) Haemocyte suspensions were incubated with laminarin or LPS and 125 μL of 0.1% NBT for 15 min at 22 °C. At the end of incubation, the reaction was stopped by adding 460 μL of 70% methanol, and the mixture was centrifuged (1500 rpm, 10 min, 4 °C). The supernatant was discarded and 4 mL of extraction fluid (2M KOH + DMSO; 6:7 ratios) were added to the pellet, to dissolve the insoluble formazan formed from NBT reduction. The samples were further centrifuged (8000 rpm, 15 min, 4 °C). The O.D. of the clear blue supernatant was measured at 625 nm using spectrophotometer, against a reagent blank consisting of 300 μL buffer, 125 μL NBT, and 4 mL extraction fluid. The values were expressed as O.D. at 625 nm/15 min.

2.5.6. In vitro phagocytosis assay

Phagocytosis assays were performed on monolayers using yeast cells as targets following Barracco et al. (1999) and Thiagarajan et al. (2006). Briefly, a sample of 50 μL haemolymph suspension was placed on a glass slide and haemocytes allowed to adhere on glass slides for 20 min at 25 °C in a moist incubation chamber. Then 50 μL of a yeast suspension (10^7 cells mL^{-1}) was added to the haemocyte monolayer, and the glass slides were returned to the moist incubation chamber for 45 min at 25 °C. After rinsing with the filtrated seawater, the slides were fixed with methanol for 5 min and stained with Giemsa solution for 20 min. Two replicates were made for each individual, and three counts of about 200 haemocytes were made for each replicate. The results were expressed as the percentage of phagocytic haemocytes.

2.5.7. Determination of lysozyme activity

The activity of lysozyme towards lyophilized cell of *M. lysodeikticus* as measured as described by Ellis (1990) and Anderson (1996). A sample of 0.5 mL plasma was mixed with 1.5 mL *M. lysodeikticus* suspension (0.2 mg mL^{-1} in 0.1 M PBS, pH 6.8) and incubated at 25 °C for 5 min, then the O.D. was measured at 1-min intervals for 5 min at 540 nm. One unit of enzyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 min^{-1} mL^{-1} serum.

2.5.8. Lysosomal membrane stability

Haemolymph (100 μL) samples were pipetted into micro centrifuge tube. Aliquots (10 μL) of 0.33% neutral red (Sigma) solution in phosphate buffered saline (0.1 M, pH 7.2 NaCl, 2%) were added to each tube and the tubes were incubated for 1 h at 10 °C. The tubes were then centrifuged for 5 min and washed twice in buffer. Aliquots (100 μL) of 1% acetic acid in 50% ethanol were added to all tubes. The tubes were covered with foil, incubated for 15 min at 20 °C and then read at 550 nm. The results were expressed as optical density per mg^{-1} mL^{-1} haemocyte protein.

2.5.9. Antibacterial activity of haemolymph

Antibacterial activity of the haemolymph was investigated by measurement of growth inhibition by turbidometry (Wootton and Pipe, 2003). Aliquots (100 μL) of haemolymph from experimental and control groups were added to 96 well plate and an equal volume of PBS was added to a control well. A log phase broth culture of *Aeromonas hydrophilla* was grown overnight in nutrient broth (NB) with constant shaking at 28 °C. A bacterial suspension (100 μL) in NB was prepared (~10^8 bacteria mL^{-1}; O.D. 600 = 0.509) and added to each of the experimental and control wells. Positive control with broth and bacteria were also maintained. Aliquots of 100 μL sterile PBS and 100 μL sterile broth were added to a well to act as a blank. The plate was incubated at room temperature and absorbance measured at 0, 1, 2 and 24 hours at 540 nm.

2.5.10. Statistical analyses

Duplicate experimental chambers were maintained for all concentrations each containing 10 abalones. Results are reported as mean ± SD of six individuals per group (3 abalone/tank) and the significance was tested. Data were compared with control groups and they were analysed by ANOVA followed by Dunnets post hoc test using SPSS 10.0 software to determine whether there is an interaction between controls and exposed. The laminarin or LPS stimulated phenoloxidase and O_2^- generation was tested for significance over the respective positive controls using a paired sample student '*t*' test (Bailey, 1959).

3. Results

3.1. Benzo(a)pyrene exposure

The 96 h sublethal (LC_0), median lethal (LC_{50}) and lethal (LC_{100}) concentration of B(*a*)P to *H. diversicolor* was found to be 0.1, 1.00 and 3.2 mg L^{-1}, respectively (Fig. 1). B(*a*)P used for all analyses was at its sublethal concentrations of 0.01–0.08 mg L^{-1}. Abalones were exposed to these concentrations for 7 d and assays performed at the end of exposure period. The behaviour of the abalone was observed to be normal as seen with normal movement, responding to stimuli and animal aggregation and no mortality was observed throughout the exposure period (7 d).

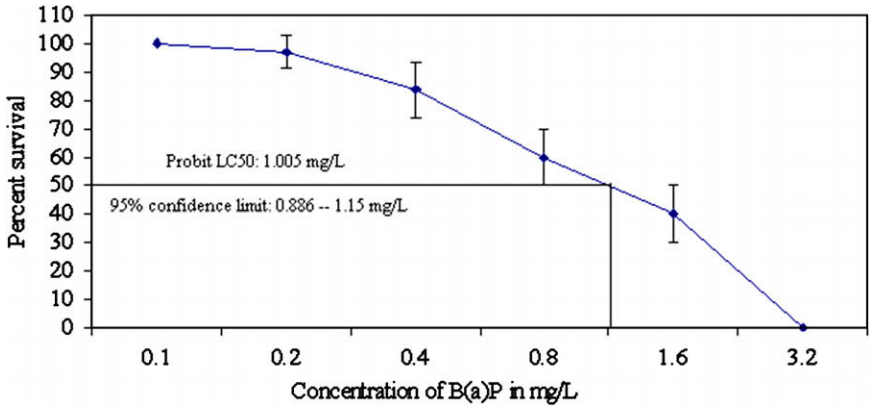

Fig. 1. Percentage survival of *H. diversicolor* in different B(*a*)P concentrations for 96 h and its median lethal concentration (LC_{50} with 95% upper and lower confidence limits) calculated by probit analysis. Control and solvent control and B(*a*)P concentration lower than 0.1 mg L^{-1} did not have any mortality until the end of the exposure periods.

3.2. Total haemocyte count (THC)

There were no significant differences in THC for the abalones kept in the control solution and solvent control after seven days of exposure. The mean (±SE) THC varied from 2.1×10^6 to 1.1×10^6 cells mL^{-1} for experimental groups. The THC of abalones exposed to 0.02, 0.04 and 0.08 mg L^{-1} of B(*a*)P decreased by 26.24%, 44.34% and 49.77%, respectively as compared to the abalones in the solvent control after 7 d of exposure. There was no significant change in the THC when abalones were exposed to solvent control and 0.01 mg L^{-1} of B(*a*)P when compared to control after 7 d (Table 1).

3.3. Phenoloxidase activity

In the present study, spectrophotometric analysis revealed the oxidation of L-DOPA by plasma of *H. diversicolor* (Figs. 2–4). Thiourea, significantly ($p < 0.01$) reduced this oxidation at a concentration of 5 mM thereby indicating the oxidation of L-DOPA by plasma PO. When plasma of *H. diversicolor* was pretreated with non-self molecules before reaction with L-DOPA, the levels of PO activity in the plasma was altered and the activation response, in particular, appeared to vary among different non-self molecules tested (laminarin or LPS). Pretreatment with laminarin (1 mg mL^{-1}), significantly ($p < 0.5$) enhanced PO activity in plasma of *H. diversicolor* thereby indicating activation response of proPO to a non-self molecule. LPS tested (0.05 mg mL^{-1}), produced activities above the buffer-incubated controls in which the enhancement obtained with LPS was statistically significant ($p < 0.5$). When using LPS or laminarin as a trigger, the phenoloxidase activity was increased in abalones exposed to 0.02 and 0.04 mg L^{-1} of B(*a*)P concentration whereas there was no significant increase in phenoloxidase activity in animals exposed to the highest concentration of B(*a*)P (Fig. 3).

The effect of sublethal concentrations of B(*a*)P on the phenoloxidase system in the plasma of *H. diversicolor* is shown in Fig. 4. No significant difference in phenoloxidase activity was observed among the abalone in the control solution and solvent control after 7 d of exposure. The higher concentrations (0.04 and 0.08 mg L^{-1}) showed increase in phenoloxidase system, wherein the levels were significantly higher ($p < 0.001$) than those obtained with control abalone. However, at 0.01 mg L^{-1} concentration of B(*a*)P there was no significant increase in PO (NS; Fig. 4), when compared to control.

3.4. Superoxide anion generation

The haemocytes from abalone for superoxide anion generation assays were divided into three different treatment groups for B(*a*)P exposed and unexposed animals. Each group consisted of unstimulated and stimulated haemocytes with laminarin (2 mg mL^{-1}) or 0.1 mg mL^{-1} LPS to check responsiveness of haemocytes from different groups to challenge by non-self molecule. As shown in Fig. 5, levels of superoxide anion in unstimulated haemocytes were significantly increased ($p < 0.05$; $p < 0.01$) in abalones exposed to B(*a*)P concentration for 7 d when compared to the control. Stimulation of haemocytes with (2 mg mL^{-1}) laminarin resulted in a significant increase ($p < 0.001$; $p < 0.05$) in superoxide anion generation (77.32% and 66.80%) in control and animals exposed to lower concentration (0.01 mg L^{-1}) of B(*a*)P and the increase in superoxide anion generation in higher concentration (0.02, 0.04 and 0.08 mg L^{-1}) were 20.47%, 16.23%, and 11.31%, respectively. Similar trend was observed when the haemocytes were stimulated with LPS, though both the stimulator

Table 1
Effect of sublethal concentrations of B(a)P on phagocytosis, THC, lysozyme activity and lysosomal membrane stability of haemocytes in *H. diversicolor*. Each bar represents mean ± SD of six determinations using samples from different preparations. The difference observed between control and B(*a*)P exposed abalones.

	Phagocytosis (%)	Total haemocyte count (no. × 10 cells mL^{-1})	Lysozyme activity (unit mL^{-1} serum)	Lysosomal membrane stability (O.D. min^{-1} mL^{-1} protein)
Control	43.16 ± 1.51	2.10 ± 0.11	14.66 ± 1.1	0.119 ± 0.011
Solvent control	39.17 ± 2.41^{NS}	2.21 ± 0.43^{NS}	12.83 ± 3.4^{NS}	0.099 ± 0.021^{NS}
0.01 mg L^{-1}	36.31 ± 1.52*	2.18 ± 0.63^{NS}	12.30 ± 1.9^{NS}	0.083 ± 0.013*
0.02 mg L^{-1}	32.11 ± 1.21**	1.63 ± 0.12*	12.0 ± 2.1^{NS}	0.080 ± 0.002*
0.04 mg L^{-1}	18.83 ± 0.98***	1.23 ± 0.41**	11.16 ± 1.8*	0.075 ± 0.012*
0.08 mg L^{-1}	16.86 ± 1.12***	1.1 ± 0.07***	5.5 ± 0.94**	0.063 ± 0.009**

NS – non-significant.
* Indicates statistically significant at $p < 0.05$.
** Indicates statistically significant at $p < 0.01$.
*** Indicates statistically significant at $p < 0.001$.

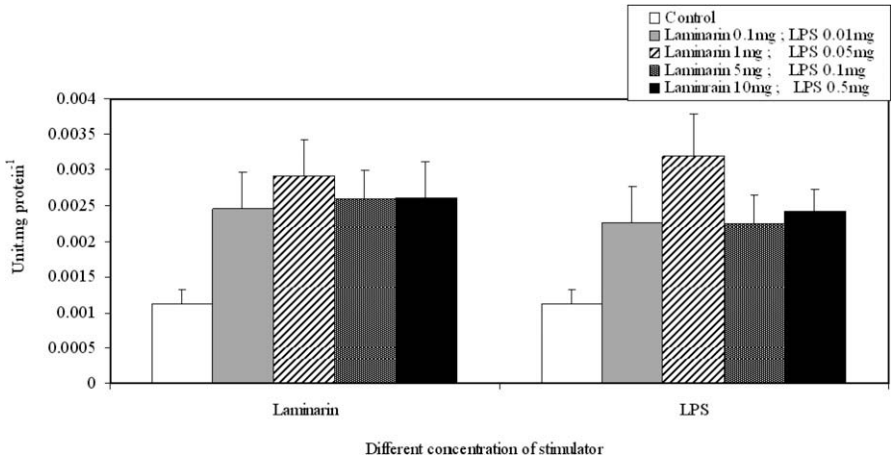

Fig. 2. Activation of prophenoloxidase in plasma of *H. diversicolor* in response to different stimulator (laminarin and LPS). Values are shown as mean ± SD of six determinations. Plasma was preincubated with laminarin (dissolved in PBS buffer, pH 7.0) or LPS at appropriate final concentrations, or as in control, with PBS buffer (pH 7.0) for 20 min prior to addition of L-DOPA.

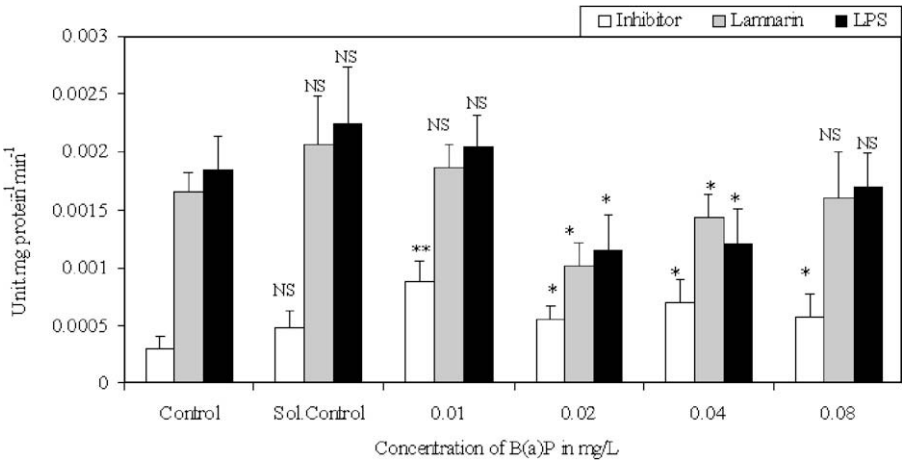

Fig. 3. Effect of sublethal concentration of B(*a*)P on the prophenoloxidase of *H. diversicolor*. Each bar represents mean ± SD of six determinations using samples from different preparations. The difference observed between control and B(*a*)P exposed abalones; * indicates statistically significant at $p < 0.05$ level for laminarin and LPS stimulator. In oxidation of L-DOPA, the difference observed between control and B(*a*)P. *, ** indicates statistically significant at $p < 0.05$, p and < 0.01, respectively. NS – non-significant.

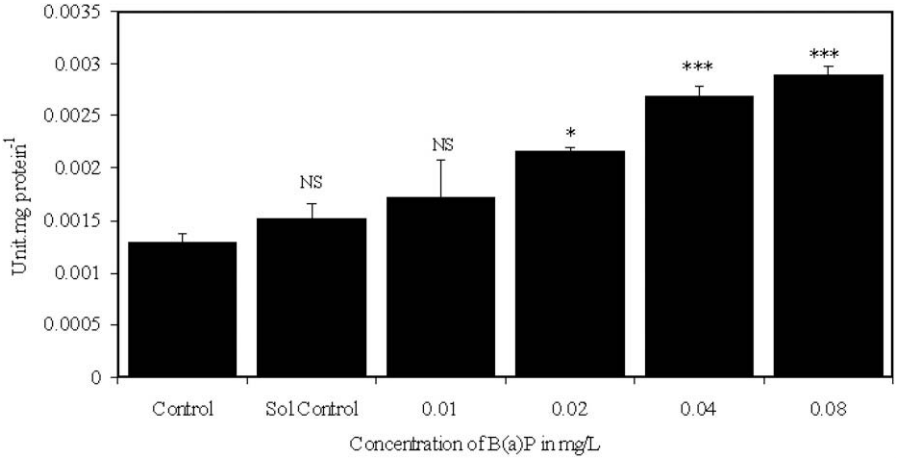

Fig. 4. Effect of sublethal concentration of B(*a*)P on the plasma phenoloxidase of *H. diversicolor*. Each bar represents mean ± SD of six determinations using samples from different preparations. The difference observed between control and B(*a*)P exposed abalones. *, *** indicates statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively. NS – non-significant.

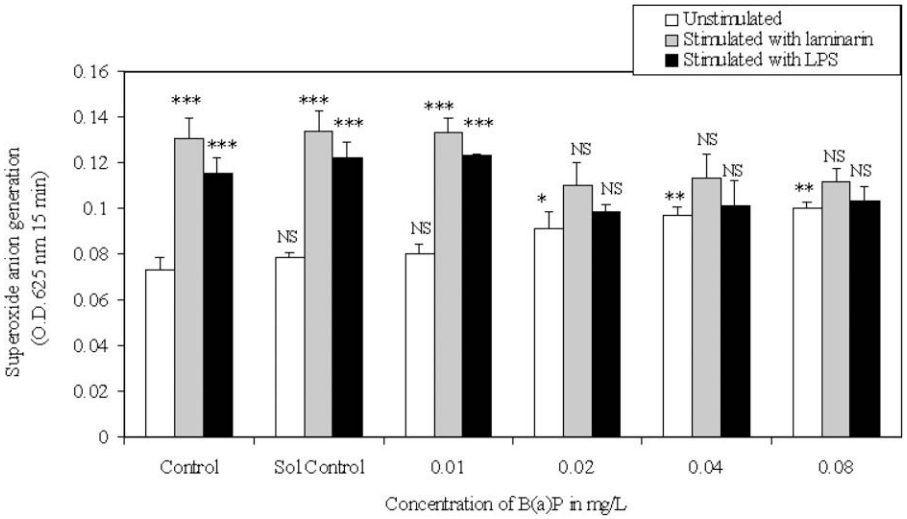

Fig. 5. Effect of sublethal concentration of B(*a*)P on the generation of superoxide anion by the haemocytes of *H. diversicolor*. Each bar represents mean ± SD of six determinations using samples from different preparations. The difference observed within the unstimulated O_2^- generation between control and B(*a*)P exposed abalones; the difference observed from the stimulated and unstimulated O_2^- generation with respect to the particular concentration exposed. *, **, *** indicates statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively. NS – non-significant.

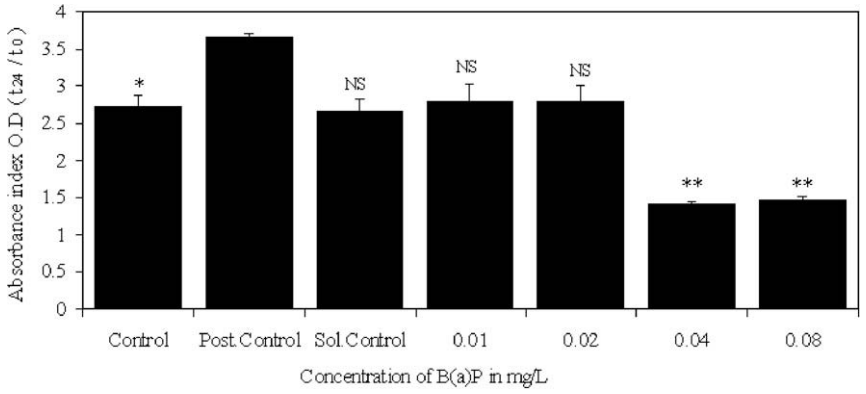

Fig. 6. Effect of sublethal concentration of B(*a*)P on the antibacterial activity of haemolymph of *H. diversicolor* after 24 h incubation. Each bar represents mean ± SD of six determinations using samples from different preparations. The positive control represents bacterial growth in broth instead of haemolypmh. The control was compared with the positive control and all other results were compared to the control. The difference observed between control and B(*a*)P exposed abalones. ** indicates statistically significant at $p < 0.01$. The difference observed between control and positive control; * indicates statistically significant at $p < 0.05$ and NS: no significant.

resulted in a significant increase, laminarin elicited a higher response than LPS.

3.5. Phagocytosis

Table 1 shows the effect of sublethal concentrations of B(*a*)P on the phagocytic ability of the haemocytes of *H. diversicolor*. As shown, higher concentrations (0.04 and 0.08 mg L^{-1}) of B(*a*)P had a deleterious effect on the phagocytic ability of the haemocytes after 7 d of exposure ($p < 0.001$) when compared to those exposed to control. After 7 d exposure, there was more than 1-fold reduction in the percent phagocytic response of haemocytes exposed to B(*a*)P at higher concentrations as compared to abalones exposed to control.

3.6. Lysosomal enzyme release

Lysosomal enzyme release by abalone haemocytes was evaluated by measuring lysozyme activity in the extracellular medium. A comparison of the results of lysosomal enzyme activity in the control and B(*a*)P exposed animal shows that the lysozyme activity decreased significantly ($p < 0.01$) at higher concentration (0.08 mg L^{-1}) of B(*a*)P tested. The effects of different concentrations of B(*a*)P on lysozyme activity of *H. diversicolor* is shown in Table 1. At lower concentration of B(*a*)P (0.01 and 0.02 mg L^{-1}) there was no significant change with respect to control.

3.7. Lysosomal membrane stability

Stability of lysosomal membranes in haemocytes of *H. diversicolor* following treatment with different concentrations of B(*a*)P was evaluated by the neutral red retention assay. The results shown in Table 1 indicate that lysosomal membrane stability was significantly reduced ($p < 0.01$; $p < 0.05$) when animals were exposed to different concentrations of B(*a*)P. As the concentration of B(*a*)P was increased in seawater the stability of the lysosomal membrane of haemocyte became weaker and the table clearly shows that B(*a*)P affected the lysosomal membrane.

3.8. Antibacterial activity

The haemolymph of abalone showed antibacterial activity (Fig. 6); however, there was high inter-individual variability. In general, both the experimental and control samples showed

bacterial growth; however, the growth was significantly greater and more rapid in the control wells, but lesser than that of positive control (bacterial growth in nutrient broth) indicating a suppression of bacterial growth in control. The bacterial growth was significantly reduced in the haemolymph collected from abalone exposed to higher concentration (0.04 and 0.08 mg L^{-1}) of B(*a*)P.

4. Discussion

Our results demonstrate that *in vivo* exposure of *H. diversicolor* to sublethal concentrations of B(*a*)P has a drastic effect on those immune parameters of the animal that were studied. Exposure to 0.04 and 0.08 mg L^{-1} B(*a*)P for 7 d resulted in a significant decrease in the number of circulating haemocytes in *H. diversicolor*. The observed decrease was statistically significant on exposure to higher concentrations of B(*a*)P, perhaps due to enhanced toxicity of the B(*a*)P causing cell death or possibly due to migration of haemocytes from the haemolymph towards areas prone to injury caused by B(*a*)P exposure. Exposure to PAHs may result in alterations in the proportions of circulating haemocytes (Sami et al., 1992, 1993). Environmental pollutants including cadmium, copper, phenol and fluoranthene affected THC in several species of molluscs (Cheng, 1988; Coles et al., 1994, 1995) and this could be due to the inhibition of mobilization of haemocytes by the xenobiotics and stressors (Ano and Mori, 1996; Vijayavel et al., 2005). It is well known that the haemocyte is the central cell of the immune defense. The important role of haemocytes in molluscs are cellular defense reactions and they are capable of chemotaxis, antigen recognition, agglutination, phagocytosis, and elimination of invaders by respiratory burst (Adema et al., 1991). A transient drop in haemocyte counts with mild stressors have been reported (Malham et al., 2003; Cheng et al., 2004a–e) which is similar to our results. The decrease in THC may reflect on other immune function such as phagocytic activity and respiratory burst of the haemocytes of abalones.

In molluscs, phenoloxidase (PO) is an important humoral defense system and it can be activated by non-self material (Asokan et al., 1997). PO is present in mollusc plasma in an inactive prophenoloxidase (proPO) state. The activation of PO leads to the melanization reaction and causes the entrapment of foreign material in a melanin capsule. Many studies have shown that PO can be released from circulating haemocytes into haemolymph when the animals are stimulated by physical injury or infestation (Asokan et al., 1997; González et al., 2003). PO usually exists as an inactive precursor, proPO, and the proPO can readily be activated to PO by the endogenous proPO-activation system or some exogenous elicitors such as laminarin, SDS, LPS etc. (Asokan et al., 1997). In our study, the proPO was detected by converting to the active PO using laminarin and LPS. The results showed that the proPO activation was greater in controls when compared to the abalone exposed to higher concentration (0.04 and 0.08 mg L^{-1}) of B(*a*)P. Changes in the level of this important defensive enzyme may effect the survival of the organisms when challenged with infectious pathogens (Thiagarajan et al., 2006).

In the present investigation, B(*a*)P had a significant effect on the plasma PO, such that an increase in the enzyme levels was found due to B(*a*)P exposure. Levels increased significantly when compared to that of control abalones and the levels were highest at higher concentration (0.08 mg L^{-1}) after 7 d of exposure. All these results suggest that the toxicity of the organic pollutant takes effect over a period of time. A similar increase in PO in *H. diversicolor* due to the effect of nitrite was observed (Cheng et al., 2004e). In contrast to our result the phenoloxidase activity decreased in *H. diversicolor* upon exposure to ammonia after 24 and 72 h exposure (Cheng et al., 2004d). An increase in PO, due to xenobiotic exposure, has been well established in molluscs (Coles et al., 1994; Thiagarajan et al., 2006) and the results of the present study also supports the earlier views, suggesting that sub-lethal levels of B(a)P have an impact on plasma PO of *H. diversicolor*. Whether increase in the level of PO activity due to B(a)P exposure would help the survival of abalones to future infection remains to be elucidated. Nappi et al. (1995) reported in his study that intermediates of PO may generate superoxide anion and thus, it is quite possible that increased levels of PO activity due to B(a)P exposure might indirectly produce free radicals that could leads to oxidative stress and cellular damage.

It has been well documented that phagocytosis is the most important and widespread immune mechanisms in both invertebrates and vertebrates and also major effector mechanism of the cellular immune component of molluscs (Pipe et al., 1999; Cheng et al., 2004a-e; Thiagarajan et al., 2006). The phagocytosis is normally used as a tool in immunotoxicological studies to evaluate the effect of toxicants on organisms and their cellular integrity (Brousseau et al., 2000; Fournier et al., 2000; Thiagarajan et al., 2006). Phagocytosis normally mobilizes lysosomes for subsequent killing of phagocytosed materials (Bayne, 1990). Lysosomes may also be involved in segregation and metabolise the toxic compounds and the presence of xenobiotics may cause membrane destabilization (Lowe and Pipe, 1994). As our results revealed that the sub-lethal concentrations of B(a)P had significant effects on the phagocytic activity of haemocytes of *H. diversicolor*. At higher concentrations (0.04 and 0.08 mg L^{-1}), B(a)P was more toxic and caused a significant reduction in percent phagocytosis when compared to the control animals after 7 d of exposure, suggesting B(a)P stress. The obtained result clearly reveals that the phagocytic activity was concentration dependent. The reduction of phagocytic activity in the present study may be due to B(a)P induced disruption in haemocyte membrane stability. As phagocytosis is mainly dependant on cell membrane functioning, any disruption in membrane may alter the process (Wooten et al., 2003).

During phagocytosis, there is an increase in oxygen consumption and an increase in production of reactive oxygen species (ROS), such as superoxide anion (O_2^-). ROS interacts with biological macromolecules and results in enzyme inactivation, lipid peroxidation, DNA damage, cell death, etc. (Cazenave et al., 2006). As known the superoxide anion generation can produce toxic metabolites and they are capable of destroying invasive pathogens. This cellular defense reaction is vital in immune response and has been well characterized in molluscs (Adema et al., 1991; Arumugam et al., 2000). Modulation in the levels of superoxide anion generation caused by xenobiotic exposure have been reported in molluscs. (Coles et al., 1995; Pipe and Coles, 1995; Cajaraville et al., 1996; Dyrynda et al., 1998; Wootton et al., 2003; Thiagarajan et al., 2006).

In the present study, B(*a*)P had a slightly stronger effect on the unstimulated generation of O_2^-, at higher concentration when compared to control haemocytes. Strikingly, the levels of O_2^- generation showed variation when non-self molecules like laminarin or LPS were used as elicitors. Significant increase in O_2^- generation was observed in control abalone upon stimulation. In contrast, abalones exposed to B(a)P showed a modulation in superoxide anion generation responses to the elicitors. Thus, these results along with dose dependency, indicate that although the haemocytes respond to B(*a*)P toxicity in terms of superoxide generation, the haemocytes can respond to a elicitors (laminarin or LPS) or a non-self challenge only at lower concentrations of B(*a*)P, and higher concentration probably affects the haemocytes' ability to respond to non-self, namely, NADPH-oxidase activation. Molluscs also contain antioxidants which will scavenges the free radicals and protect the animals from oxidative stress. However, it is not known whether increase in generation of free radicals in animal is due to

augmented production or suppression of antioxidant enzymes which may reflect the cellular damage (Martello and Tjeerdema, 2001).

Lysosomal membrane stability assay using neutral red has been proposed as a useful integrative biomarker in immunotoxicological study. The supravital dye will be retained only in the lysosomes of healthy cells after its initial uptake (Lowe and Pipe, 1994). The quantification of neutral red assay using spectrophotometer has been correlated with the number of viable cells (Borenfreund and Puerner, 1985). In addition, this rapid assay method was more easy for the estimation of cellular density compared with direct counting in the microscope. Both chemical and non-chemical stressors including hypoxia, hyperthermia, osmotic shock and nutritional deprivation affect lysosomal membrane stability, suggesting that haemocyte viability itself is central to the response to both immunological challenge and other stressors (Galloway and Depledge, 2001). In the present study the stability of haemocyte lysosomal membranes following exposure to different concentrations of B(*a*)P was evaluated and there was a drastic reduction when abalones were exposed to higher concentration of B(*a*)P. In a study of the immunotoxic effects of a cocktail of PAH in *M. edulis* (Grundy et al., 1996), both phagocytosis and lysosomal integrity were inhibited when the mussels were exposed to environmentally realistic concentrations of the PAH mixture for 2–4 weeks illustrating the potential impact of PAH on lysosomal membrane damage in these animals which leads to alter the immune function in *M. edulis*. In the present study the haemocyte lysosomal membrane injury was evident using the neutral red probe at B(*a*)P exposure and the elevated membrane permeability was likely to increase autophagic events and hydrolytic enzyme activity in the cytosol leading to a greater risk of cytotoxicity. As described by Sminia, the molluscs immune response is comprised of an integrated process of phagocytosis and lysosomal degradation (Sminia, 1980) and pollution induced dysfunction of these processes may suppress immunocompetence.

In the immune system of molluscs, lysozyme is one of the most important bacteriolytic agents against several species of Gram-positive and Gram-negative bacteria (Cheng and Rodrick, 1974). During phagocytosis, the haemocytes produce lysosomal enzyme called lysozyme which actively participate in the inactivation of invading pathogens. Reduced lysozyme activity, therefore, suggests immunosuppression, resulting in lowered resistance to bacterial challenge. The results of our study revealed that lysozyme activity was higher in control abalone compared to B(*a*)P exposed but there is no significant change between the lysozyme activity of abalone exposed in the control and lower concentration of B(*a*)P. These differences in results may be due to the effect caused by different doses of B(*a*)P on abalone. A concentration-dependent lysosomal destabilization and the reduction of lysosomal enzyme release were observed in the present study, which was well related with the decrease of THC. In this study, significant inhibition of lysozyme activity was observed in abalone exposed to higher concentration of B(*a*)P and a decrease in phagocytic activity occurred at the highest concentration tested reflected the decrease in lysozyme activity. These results supported the previous data concerning the capability of lipophilic contaminants to alter enzymatic activity in fish cells (Brüschweiler et al., 1996) and bivalve haemocytes (Cima et al., 1999; Matozzo et al., 2002).

Antibacterial activity has previously been clearly demonstrated in a wide range of molluscs species (Anderson and Beaven, 2001). In the present study antibacterial activity of haemolymph is shown in Fig. 6. Both the experimental and control haemolymph showed antibacterial activity. The results demonstrated that there was a significant decrease in bacterial growth in the haemolymph collected from the control abalone when compared to positive control and this clearly reveals the presence of antibacterial activity in the haemolymph of control abalone. Surprisingly, the growth of bacteria was significantly slower in the haemolymph from abalones treated with higher concentration (0.04 and 0.08 mg L^{-1}) of B(*a*)P and no significant difference was found between the experimental groups treated for lower concentration (0.01 and 0.02 mg L^{-1}) and control.

In conclusion, our preliminary studies on abalones with B(*a*)P concentrations ranging from 0.001 to 0.010 mg L^{-1} showed no detectable changes; hence, further analyses were carried out with B(*a*)P concentrations starting from 0.010 mg L^{-1}. Since the environmental concentrations of B(*a*)P in the local coastal area was around 0.56–3.32 μg L^{-1} in the surface water from the Jiulong River Estuary and Western Xiamen Sea (Maskaoui et al., 2002), such higher concentrations used in the animals tested are quite possibly caused by bioaccumulation. The results of the present study show that short-term exposure to B(*a*)P, at sublethal concentration significantly influenced various aspects of immune function in abalones. Such experiments may bring more knowledge on the effects of pollutants on animals and how they possibly adapt to environmental xenobiotics in natural systems. The immune system in marine organisms is subject to change in various seasons, hence, this analysis is not definitive in terms of conclusion. Moreover, haemocyte populations, habitat of animals, and other environmental factors, all of which will together have a profound influence on the overall animal responses to xenobiotics. The overall results suggest that immunotoxicological effects of B(*a*)P followed a concentration-dependent response relationship. The variability in the nature and degree of immunomodulation in different aspects of internal defence emphasizes the need for a multi-assay approach to the assessment of immunocompetence.

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