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Effects of tributyltin and benzo[a]pyrene on the immune-associated activities of hemocytes and recovery responses in the gastropod abalone, *Haliotis diversicolor*

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ABSTRACT

Our previous study reports that short-term exposure to sublethal concentrations of benzo[a]pyrene (BaP) induces immunomodulation in the gastropod abalone, *Haliotis diversicolor*. In the present study, it was further observed that long-term chronic exposure to sublethal concentrations of BaP modulated the immunocompetence of abalones in terms of the change in activity of the antioxidant and immune associated parameters tested. In addition, the effect of tributyltin (TBT), another important genotoxicant in the aquatic environment, was investigated. Exposure of abalones to sublethal concentrations of TBT and BaP for 21 days resulted in significant decrease of total hemocyte count, phagocytosis, membrane stability and lysozyme activity. Conversely induction of extra and intra cellular superoxide generation, nitric oxide, nitric oxide synthase and myeloperoxidase activity was present when the abalones were exposed to TBT and BaP. Most of the immune associated parameters tested showed clear time dependent response to both toxicants. Within 14 days after the 21 day exposure to BaP, recovery was observed as evidenced by most of the parameters returning to their normal level. However, no recovery was observed within 14 days after the 21 day exposure to TBT as evidenced by continued elevation of intra cellular superoxide and nitrite production and decrease in THC, membrane stability and lysozyme activity. This suggested a prolonged TBT-induced impact on the immune reaction and possibly more damage than that caused by BaP. Overall the results suggest that chronic exposure to sublethal concentrations of TBT or BaP causes modulations in the immunocompetence of abalones with most of the immune associated parameters tested being stimulated, and this might be harmful to the host.

1. Introduction

Xenobiotics such as tributyltin (TBT) and benzo[a]pyrene (BaP) are major anthropogenic contaminants which have been released into aquatic ecosystems, and this activity is not expected to change in the foreseeable future (Petridis et al., 2009). Hence it is necessary to have a clear understanding concerning the fate and effects of these contaminants in aquatic animals, especially those in coastal regions which may be involved in the human food chain. TBT is a highly toxic organometallic compound used worldwide as an active biocide and catalyst (Oberdorster et al., 1998). However, this biocide is now restricted in its use as the active component of anti-fouling paints in various countries (Evans et al., 1995) and, moreover, the International Marine Organization has imposed a worldwide ban on TBT usage in marine applications (Bekri et al., 2006) due to its adverse effects on marine organisms. BaP is a widespread carcinogenic polycyclic aromatic hydrocarbon, which has been reported to be a mutagenic and carcinogenic precursor. Both these compounds can produce embryo toxicity (Marin et al., 2000; Wessel et al., 2007) and genotoxicity (Jha et al., 2000), and they are also reported to be strong immunomodulators (Anderson et al., 1997; Cima et al., 1998, 1999; St-Jean et al., 2002a; Gopalakrishnan et al., 2009).

The immune defense system of mollusks mainly depends on innate immunity and more specifically on circulating hemocytes, which are also thought to be important antimicrobial effector cells in mollusks and other invertebrates. One of the important roles of hemocytes in the invertebrate defense system is phagocytosis. Since this is an early internal defense mechanism against invaders by the circulating hemocytes, any decrease in total hemocyte count (THC) and phagocytic activity due to xenobiotic chemicals could lead to a decrease of the defense response against pathogens (Hooper et al., 2007; Gopalakrishnan et al., 2009; Yue et al., 2010). Hemocytes also play important roles in lysosomal enzyme activity, anti-inflammation, wound repair and the production of reactive oxygen species (ROS). In vertebrates, nitric oxide (NO) is a molecular mediator for non-specific immune responses and its production in response to microbial infection has been reported also in invertebrates (Conte and Ottaviani, 1995). The NO generating system might be a sensitive biomarker of stress in invertebrates but, in mollusks, the response of this system to chronic pollutant contamination is poorly understood (Smith et al.,

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2000). Myeloperoxidase (MPO) is a peroxidase enzyme, most abundantly present in the leukocyte components in vertebrate species and they are able to generate potent microbicidal substances. It is reported that molluskan species have MPO-like activity, which is thought to serve a similar function to that of vertebrate leukocytes (Nakamura et al., 1985; Schlenk et al., 1991). MPO is also capable of transforming xenobiotics to free radicals (Ritter and Malejka-Giganti, 1989). Although this MPO-like activity is reported in several invertebrate species (Nakamura et al., 1985; Schlenk et al., 1991), the effect of xenobiotics on the MPO-like activity has not been covered.

However, several studies report that invertebrate immune functions are adversely affected by xenobiotic exposure (Pipe and Coles, 1995; Anderson et al., 1997; Matozzo et al., 2003; Gopalakrishnan et al., 2009; Zhang et al., 2009). Furthermore, it is noted that exposure of aquatic invertebrates to environmental contaminants such as TBT and BaP modulate the hemocytic defense mechanism (Fisher et al., 1990; Anderson et al., 1996; Gopalakrishnan et al., 2009; Zhou et al., 2010). Cellular immune function and defense impairment due to TBT are observed in oyster (Auffret and Oubella, 1997) and also TBT inhibits NADPH cytochrome C-reductase activity in mollusks (Anderson et al., 1997; Morcillo and Porte, 1997). Previous studies reveal that the immunotoxicity of TBT appears to induce extracellular superoxide generation, decrease phagocytic ability, and further suppress the non-specific immune competence (Anderson et al., 1997; Regoli et al., 2002; Gagnaire et al., 2006).

Among the gastropods, the large and algivorous abalones are one of the most important commercial species in coastal aquaculture. A few studies report that environmental stressors (including xenobiotics) have negative impacts on the abalone immune system, resulting in an increase in disease susceptibility (Jia et al., 2009a; Morley, 2010). Zhou et al. (2010) report that TBT at concentrations ranging from 2 to 50 ng/L impairs abalone immunological ability and is a potential immune disruptor. Previous studies report that TBT and BaP exposure can modulate the generation of ROS in mollusks and result in a certain degree of immune system impairment, but these studies were mostly restricted to short term or in vitro exposures (Anderson et al., 1997; Matozzo et al., 2003; Gopalakrishnan et al., 2009; Zhang et al., 2009).

The objectives of the present study were to determine firstly whether exposure of abalones to sublethal concentrations of TBT and BaP would induce physiological changes in various immune-associated parameters and secondly the progress of the effects on *H. diversicolor* during a recovery study. This investigation is the first to evaluate the immunocompetence of abalones under sublethal continuous exposure to TBT and BaP for 21 days followed by a 14 day recovery period. Finally, the different functional parameters of hemocytes were evaluated and the possible role of ROS in mediating the impact of TBT and BaP was investigated.

2. Material and methods

2.1. Animals

Normal abalones (*Haliotis diversicolor*; 60 ± 10 mm in shell length) without discernable injury or any parasites were obtained from the Zhangpu abalone farm of Fujian Province, China and were acclimatized under laboratory conditions with a temperature of 25 ± 1 °C, salinity of 30 ± 1‰ and pH of 7.8 ± 0.1 for 10 days before experimentation.

2.2. Exposure conditions

Ninety three abalones were used for the immunotoxicity biomarker study. They were divided into three groups. Group I were exposed to 0.35 μg L^{-1} of TBT; group II to 0.05 mg L^{-1} of BaP; and group III to acetone (the solvent control; $V_{acetone}/V_{seawater} = 1/20{,}000$). Duplicate chambers were maintained for the exposed groups and the solvent control. In addition, three abalones were maintained in seawater separately and used as the normal control. Sampling was performed at different time intervals (0, 3, 7, 14 and 21 days) during the exposure period. A 14 day recovery period (after the 21 days of toxicant exposure) was also used and sampling was at intervals of 7 days during this recovery study. Each treatment for each toxicant was carried out in duplicate, with replicate treatments carried out on different days. Since the half life period of BaP and TBT are comparatively long (ATSDR, 1990; USEPA, 2003), approximately 90% of the test solution and seawater was renewed daily (EPA/ROC, 1998) up to 21 days of exposure and, during the recovery study only seawater was used instead of the test solution. The abalones were fed with *Gracilaria tenuistipitata* during the experiment.

2.3. Isolation of plasma and hemocytes and preparation of hemocyte lysate suspension

The methods for collection of hemolymph, isolation of plasma and separation of hemocytes followed the same procedure as that described in our previous study (Gopalakrishnan et al., 2009). Hemocytes were subjected to cell disruption (20 kHz, 50 W, 3×20 s) in an ultrasonicator (Scientz JY92-II, Ning Bo Xinzi Company) and the resultant homogenates were centrifuged ($12{,}000 \times g$, 30 min, 4 °C). An aliquot of the resulting clear hemocyte supernatant (HLS) from each sample was used for the determination of NO production.

2.4. Immune associated parameters

2.4.1. Total hemocyte count

THCs (number of cells mL^{-1} hemolymph) were made with an improved Neubauer hemocytometer using fixed hemolymph samples. A sample of 25 μL of hemolymph was added to a hemocytometer and counted microscopically under 40× magnification.

2.4.2. Detection of intracellular superoxide anions

The intracellular generation of superoxide anions (O_2^-) by the hemocytes (control and toxicant exposed) of abalone was assessed using nitro blue tetrazolium following the procedure of Arumugam et al. (2000) as described in detail in our previous study (Gopalakrishnan et al., 2009).

2.4.3. Detection of extracellular superoxide anion

The generation of extracellular superoxide was measured using the reduction of cytochrome-c following Dyrynda et al. (2000) with slight modification. Hemolymph from each abalones was pipetted into a 96-well plate with an equal volume of cytochrome-c solution (80 mM ferricytochrome-c in phosphate buffer saline (PBS) (0.1 M, pH 7.4)), and cytochrome-c solution plus SOD (300 units mL^{-1}) was added to the remaining three wells. Control wells, in triplicate, included additional hemocyte aliquots plus PBS and cytochrome-c (±/SOD) plus PBS. The plates were read at 550 nm at 0 and 30 min in a microplate reader and the results were expressed as the change in the optical density (OD) per mg protein.

2.4.4. Nitrite production

Production of NO by abalone hemocytes was evaluated as described previously (Tafalla et al., 2002) using the Griess reaction, which quantifies the nitrite (NO_2^-) content of HLS. Aliquots (100 μL) of HLS were incubated for 10 min in the dark with 100 μL of 1% (w/v) sulphanilamide in 5% H_3PO_4 and 100 μL of 0.1% (w/v) N-(1-naphthy)-ethylenediamine dihydrochloride. The absorbance of the samples was measured at 540 nm in a spectrophotometer against a suitable reagent blank. The molar concentration of nitrite in the samples was determined from a standard curve generated using known concentrations of sodium nitrite and was represented as μM nitrite.

2.4.5. Nitric oxide synthase (NOS)

The NOS activity in HLS was measured using a Diagnostic Reagent Kit purchased from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer's instructions. Activities of NOS were referred to their total enzyme form.

2.4.6. MPO activity

The MPO activity in the HLS was measured according to the method described by Schlenk et al. (1991) with slight modification. Briefly, 50 mM sodium acetate buffer (pH 5.0) and 0.88 mM 3,3′,5,5′-tetra methylbenzidine with 5 mM H_2O_2 (final concentration) was added to a 96-well plate. The reaction was initiated by adding the sample and was monitored continuously at 655 nm. Results were expressed as changes in OD per mg^{-1} mL^{-1} hemocyte protein.

2.4.7. In vitro phagocytosis assay

The phagocytosis assay was performed on monolayers using yeast cells as targets following Thiagarajan et al. (2006). Hemolymph suspension (50 μL) was placed on a glass slide and the hemocytes allowed to adhere to the glass slide for 20 min at 25 °C. Subsequently, 50 μL of yeast suspension was added and the glass slides were further incubated for 45 min at 25 °C. After rinsing with filtrated seawater, the slides were fixed with methanol for 5 min and stained with Giemsa solution for 20 min. Replicates were made for each abalone, and three counts of approximately 200 hemocytes were made for each replicate. The results were expressed as the percentage of phagocytic hemocytes.

2.4.8. Lysosomal membrane stability

Membrane stability was measured as described previously by Gopalakrishnan et al. (2009). Briefly, hemolymph samples were pipetted into micro centrifuge tubes with neutral red solution in PBS and the tubes were incubated for 1 h at 10 °C. The tubes were then centrifuged for 5 min and washed twice in PBS. Aliquots of 1% acetic acid in 50% ethanol were added to all tubes. The tubes were covered with foil and further incubated for 15 min at 20 °C and then read at 550 nm. The results were expressed as OD per mg^{-1} mL^{-1} hemocyte protein.

2.4.9. Lysosomal enzyme release

Lysosomal enzyme release by abalone hemocytes was evaluated by measuring lysozyme activity in the extracellular medium. Briefly, the lysozyme activity in aliquots of plasma were measured utilizing *Micrococcus lysodeikticus* as described by Hutchinson and Manning (1996). The reduction in absorbance at 450 nm was determined over a 10 min period at 28 °C in a microplate reader. Hen egg white lysozyme, with a specified activity of 46,200 units mg^{-1} was used as the external standard. The amount of lysozyme present in the serum was calculated based on the standard.

2.5. Statistical analyses

Statistical comparisons were performed using a two-way analysis of variance (ANOVA) and SPSS software (Ver 10.0; SPSS). Briefly, duplicate experimental chambers were maintained for all concentrations, the results reported as mean ± S.D. of six individuals per group per time point (3 abalones/tank) and the significance tested. The data were first tested for normality and homogeneity using Bartlett's test. Since all data were normal, we then determined, using ANOVA, whether the groups differed and if the ANOVA-calculated p value was significant ($p \leq 0.05$). Tukey's multiple-comparison post hoc test was performed to identify statistical differences between exposed groups and solvent control groups (Zar, 1999). Principal component analysis (PCA) and a correlation matrix were used to assess the interrelationships among the parameters used. "Varimax Rotation" was used for extraction and deriving factors in the PCA and the Pearson correlation coefficient was used in the correlation matrix. Differences were statistically significant when $p < 0.05$ and 0.01, respectively. There was no significant difference between the solvent and blank controls, and therefore only the results of the solvent control are shown in the figures.

3. Results

3.1. Total hemocyte counts

Both TBT and BaP significantly reduced THC during the exposure periods. TBT exposure resulted in a decrease in THC throughout the exposure period. Similarly BaP exposure resulted in a significant decrease in THC except at 3 days. Both TBT and BaP decreased THC by a 1 fold reduction after 14 days of exposure. After the 14 day recovery period, BaP exposed abalones showed no significant change in the THC when compared with the respective control group. However, the TBT exposed group still exhibited a significant decrease in THC during the recovery period (Fig. 1).

3.2. Phagocytic ability

Phagocytic ability in the control abalone hemocytes showed no change between different exposure periods. Abalone exposed to TBT showed significant decrease in phagocytic activity throughout the exposure period and the mean (±SE) phagocytosis rate varied from 31% to 22%. However, BaP exposed abalone showed decrease in phagocytic activity only after 7 days of exposure and the mean (±SE) phagocytosis rate varied from 39% to 24%. During the recovery period both TBT and BaP exposed abalones showed significant decrease in phagocytic ability (Fig. 2). Though the phagocytosis rate in BaP exposed abalones increased from 24% (after 21 days of exposure) to 33% (after 14 days of recovery) it was still significantly decreased when compared to the respective control group.

3.3. Intra and extra cellular superoxide

When the abalone were exposed to TBT, the intracellular generation of superoxide was significantly induced (46, 45, 52 and 48% for 3, 7, 14 and 21 days; $p < 0.05$) with respect to the control group throughout the exposure period. Except at 3 days, abalones exposed

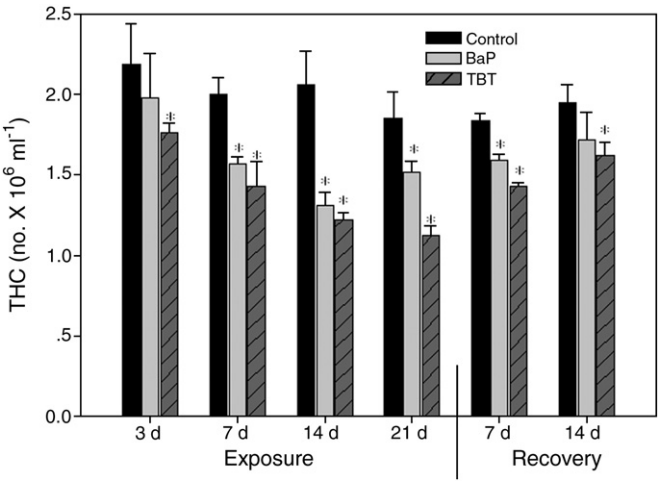

Fig. 1. Effect of sub-lethal concentrations of TBT and BaP on THC in *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p < 0.05$).

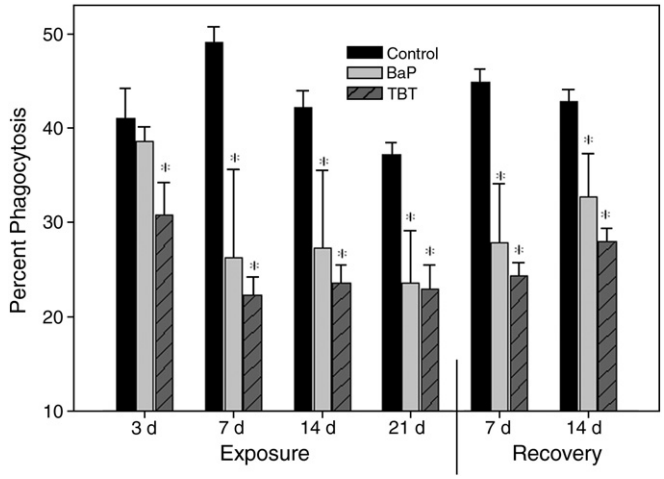

Fig. 2. Effect of sub-lethal concentrations of TBT and BaP on the phagocytic response of hemocytes of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

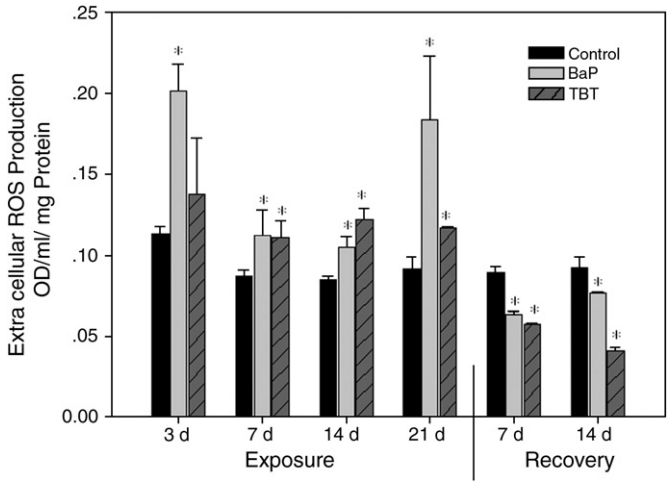

Fig. 4. Effect of sub-lethal concentrations of TBT and BaP on extra cellular generation of superoxide anions by hemocytes of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

to BaP showed intracellular superoxide generation throughout the exposure period (38, 33 and 30% for 7, 14 and 21 days; $p<0.05$). During the recovery study, BaP exposed abalones showed no significant change in intracellular superoxide generation compared with the control group throughout the 14 days (Fig. 3). Conversely, TBT exposed abalones still showed significant induction of intracellular superoxide generation. The extra-cellular release of superoxide (indicated by cytochrome-c reduction) showed significant induction during the exposure period when abalones were exposed to BaP: the maximum induction was observed after 3 days (44%) and 21 days (50%) of exposure. TBT exposed abalones showed significant induction in extra cellular superoxide after 7 days of exposure. Conversely, both TBT and BaP exposure groups showed significant reduction in extra cellular release of superoxide during the recovery period (Fig. 4).

3.4. NO generation and NOS activity

Initial results showed that abalones exposed for 3 days to both TBT and BaP showed no significant change of NO content. However, TBT exposure significantly induced NO content in *H. diversicolor* after 7 to 21 days of exposure (nearly 4 to 6 fold) (Fig. 5). However, BaP resulted in significant induction of NO (nearly 4 fold) only after 14 and 21 days of exposure. Throughout the 14 day recovery period only TBT exposed groups still showed significant induction of NO content compared to the respective control group, whereas the NO contents in BaP exposed abalones returned to the control level. The NOS activity in the hemocytes cellular fraction showed no change up to 7 days of exposure in either group, but after 14 and 21 days of exposure, both TBT (3 and 8 fold) and BaP (1 and 3 fold) showed significant induction in NOS activity. The NOS activity in both groups remained at a high level compared to the respective control group after 7 days of recovery (5 fold for the BaP exposed group and 9 fold for the TBT

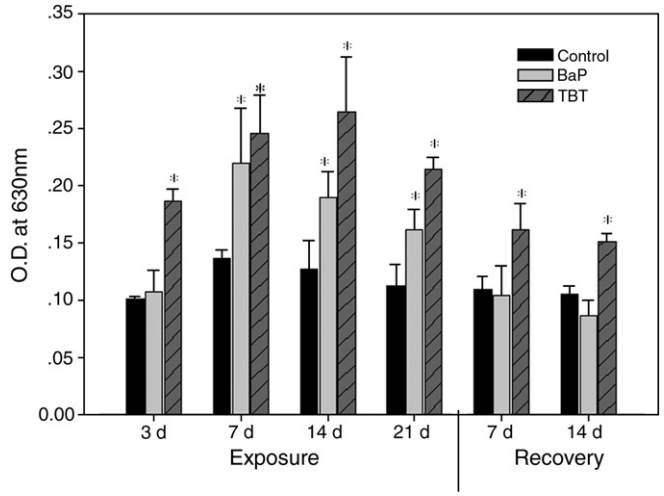

Fig. 3. Effect of sub-lethal concentrations of TBT and BaP on intra cellular generation of superoxide anions by the hemocytes of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

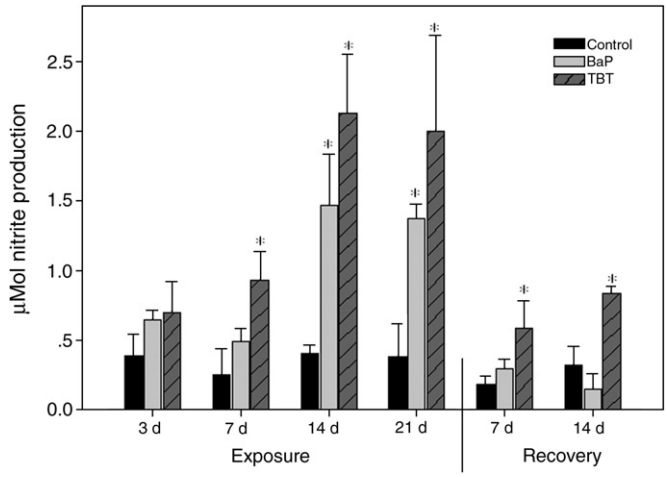

Fig. 5. Effect of sub-lethal concentrations of TBT and BaP on nitrite production by HLS of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

exposed group). Interestingly, the NOS activity in both groups returned to the control level after 14 days of recovery (Fig. 6).

3.5. MPO activity

MPO activity in abalones exposed to TBT and BaP was induced after 3 days; however such induction showed no significant change compared with the respective control group. After prolonged exposure (14 days), MPO activity was significantly induced (55% for the BaP exposed group; 58% for the TBT exposed group; $p<0.05$) in *H. diversicolor* (Fig. 7). In addition, TBT showed significant induction of MPO activity (42%) after 21 days of exposure. During the recovery period both the TBT and BaP exposed groups showed no significant change in MPO activity compared with the respective control group after 7 days. However after the 14 day recovery period TBT exposed abalones showed a decrease in MPO activity compared with the respective control group (Fig. 7).

3.6. Lysosomal membrane stability and lysozyme activity

The effect of TBT and BaP on lysosomal membrane stability was studied in order to test cellular toxicity on abalone hemocytes. Significant decrease in membrane stability was observed after 7–21 days of exposure in both the BaP and TBT groups. After the 14 day recovery, the membrane stability of BaP exposed abalones returned to the respective control level, however the TBT exposed group still showed significant decrease in membrane stability compared with the respective control group (Fig. 8). BaP significantly induced lysozyme activity in abalones after 3 days of exposure but, after 7 days of exposure, both TBT and BaP reduced the lysozyme activity (BaP 61%; TBT 75%) in *H. diversicolor* significantly compared with the respective control group. Decreases in lysozyme activity were also observed at 14 and 21 days of exposure (BaP 76 and 41%; TBT 40 and 65% reduction in lysozyme activity compared with the respective control group) (Fig. 9); during the recovery period the lysozyme activity in BaP exposed abalones returned to the normal respective control level. However, for the TBT exposed group, the lysozyme activity increased after 7 days of recovery but then decreased significantly after 14 days of recovery.

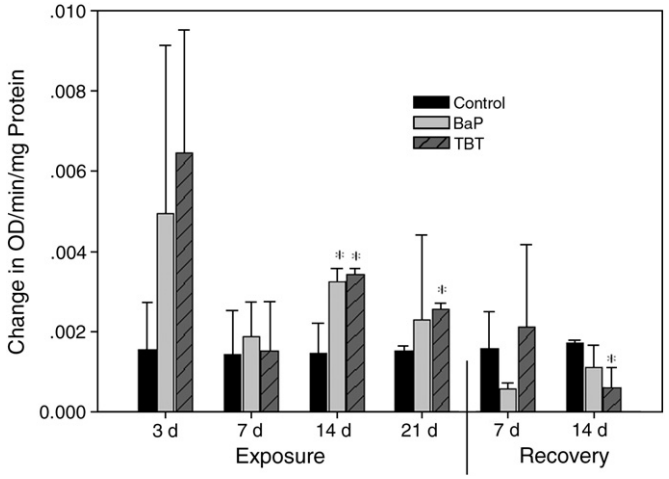

Fig. 7. Effect of sub-lethal concentrations of TBT and BaP on MPO activity by HLS of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

3.7. Correlation analysis

The calculated Pearson correlation matrix is given in Table 1. The correlation among the markers produced similar results to those of the PCA and showed significant ($p<0.01$; $p<0.05$) association between the parameters. There were good correlations between THC and the other parameters (except for the MPO and extracellular superoxide anions) studied, and the coefficients of correlation in most cases were greater than 0.599. Phagocytosis was highly correlated with THC and membrane stability; similarly it showed a positive correlation with extra cellular superoxide and negative correlation with NOS. Superoxide anions generated in the hemocytes showed a significant relationship with NO production. Although there was a relationship observed between MPO, intracellular and extracellular superoxide it did not show any statistical significance.

The rotated component matrix, developed by PCA on the measured parameters, is given in Table 2. The dimensions of the parameters were reduced from nine original variables to three principal components

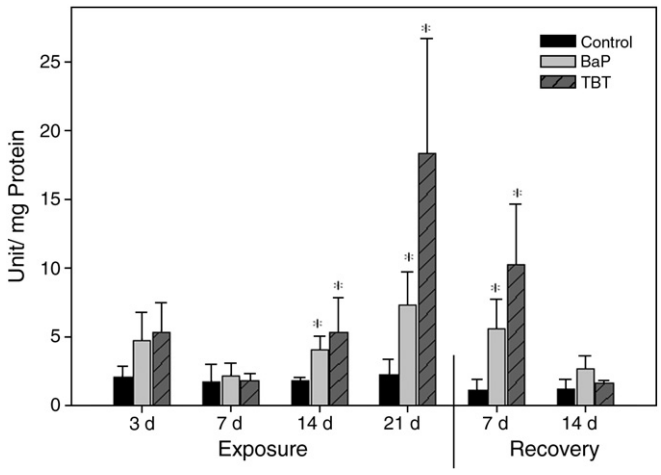

Fig. 6. Effect of sub-lethal concentrations of TBT and BaP on NOS activity by HLS of *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

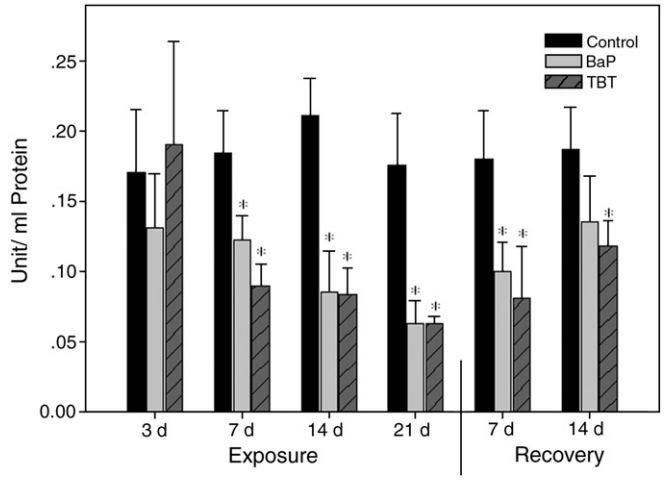

Fig. 8. Effect of sub-lethal concentrations of TBT and BaP on lysosomal membrane stability of hemocytes in *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

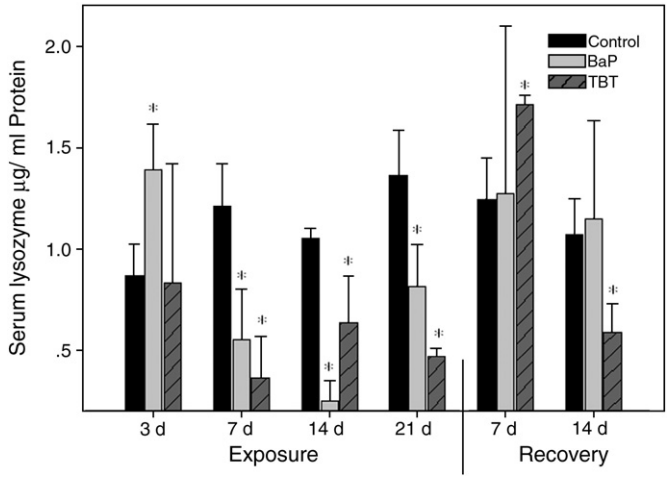

Fig. 9. Effect of sub-lethal concentrations of TBT and BaP on lysozyme activity of hemocytes in *H. diversicolor*. Each bar represents mean ± standard deviation of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The significant difference between control and exposure groups is indicated with asterisks (*: $p<0.05$).

using PCA with an eigen value >1.0. Results showed that approximately 85% of the variations were accounted for by the first three components. Of this variation, component 1 accounted for 55.22% of the variation, while components 2 and 3 accounted for approximately 18 and 12% of the variation.

4. Discussion

The present study was designed to test the toxic effects of genotoxic compounds at sublethal levels during long term exposure followed by a recovery period. The concentrations of TBT and BaP used for the present investigation were within the range of concentrations found in different contaminated environments (Catallo and Gambrell, 1987; Gabrielides et al., 1990; Michel and Averty, 1999). Both of the concentrations used are reported as sublethal concentrations in earlier studies using the same species, and these concentrations did not produce any mortality throughout the exposure periods (Jia et al., 2009b; Gopalakrishnan et al., 2009). The study involved several exposure time points (0, 3, 7, 14 and 21 days), however for the first time point (0 day) we could not observe any significant differences between the blank control and the exposure groups in any of the parameters analyzed, and so the results of day 0 were not presented. Furthermore, an earlier study reports that the immune function in the scallop recovered to the normal level after 7 days of the recovery period following exposure to PAH for 15 days (Hannam et al., 2009), and so a 14 day recovery period was chosen for the present study following the 21 day exposure.

Table 2
Rotated component matrix developed by principal component analysis for *H. diversicolor* exposed to BaP and TBT (only value >0.40 are listed here).

	Component		
	1	2	3
NOS	−0.887	–	–
Membrane stability	0.856	–	–
THC	0.806	0.548	–
Phagocytosis	0.754	0.528	–
NO	−0.626	−0.584	–
Intracellular superoxide	−0.405	−0.795	–
Lysozyme	–	0.927	–
MPO	–	–	0.907
Extracellular superoxide	–	–	0.893
Eigen value	4.971	1.66	1.05
Cumulative percentage	55.22	73.71	85.44

It is supposed that the invertebrate defense system depends solely on an innate immune system in which the circulating hemocytes play key roles and, more importantly, that THC can reflect the health status of the host. Earlier studies show that hemocyte functions can be applied as useful biomarkers to study the impact of pollution (Dyrynda et al., 1998; Fisher et al., 2000; Thiagarajan et al., 2006; Gopalakrishnan et al., 2009). In the present study THC decreased as the exposure time increased and TBT exposure significantly reduced the THC even during the recovery period. Previous studies using bivalve mollusks report that THCs are significantly increased with phenanthrene exposure (Hannam et al., 2010) and that abalones exposed to BaP show a decrease in THC (Gopalakrishnan et al., 2009). These modulations in the THC counts suggest that PAH exposure may cause cytolysis in lysosome-enriched cells such as hemocytes (McCormick-Ray, 1987) as cited in Hannam et al. (2009). After the 14 day recovery period, the abalone previously exposed to BaP showed no significant change of THC compared with the respective control group, implying that the animal was able to recover from BaP toxicity. In addition, the return of hemocyte counts to the normal level after the recovery period implies the stimulation of cell production (Pipe et al., 1999) in abalone. However TBT showed a more persistent effect on circulating hemocyte numbers in abalone.

Exposure of abalones to both TBT and BaP significantly reduced phagocytic activity during the exposure period. The results obtained clearly revealed that the phagocytic activity was exposure time dependent. These results are similar to those reported in earlier studies involving exposure of *M. edulis* to TBT (St-Jean et al., 2002a,b) and abalones to BaP (Gopalakrishnan et al., 2009) and TBT (Zhou et al., 2010). Phagocytosis is dependent on the membrane properties of the hemocytes (Grundy et al., 1996) and undergoes modulation on

Table 1
Correlation matrix for measured parameters in *H. diversicolor* exposed to BaP and TBT.

		Intracellular superoxide	Extracellular superoxide	NO	NOS	THC	Phagocytosis	MPO	Lysozyme	Membrane stability
Correlation	Intracellular superoxide	1.000	0.186	0.754*	0.337	−0.781*	−0.699*	0.308	−0.681*	−0.551*
	Extracellular superoxide		1.000	0.351	0.186	−0.040	−0.067	0.672*	−0.154	−0.156
	NO			1.000	0.613*	−0.803*	−0.655*	0.349	−0.632*	−0.699*
	NOS				1.000	−0.638*	−0.533**	0.258	−0.090	−0.611*
	THC					1.000	0.894*	−0.147	0.550*	0.863*
	Phagocytosis						1.000	−0.116	0.530**	0.864*
	MPO							1.000	−0.111	0.005
	Lysozyme								1.000	0.396
	Membrane stability									1.000

* Correlation is significant at the 0.01 level.
** Correlation is significant at the 0.05 level.

exposure to organic contaminants. Moreover, the immunotoxicity of TBT and BaP for aquatic organisms involve significant inhibition of phagocytosis (Cima et al., 1998; Gomez-Mendikute et al., 2002; St-Jean et al., 2002a,b; Gopalakrishnan et al., 2009). As reported, TBT (Cima et al., 1998; Cima and Ballarin, 2000), and BaP (Gomez-Mendikute et al., 2002; Gopalakrishnan et al., 2009) exert their toxicity by means of direct or indirect interactions with the cytoskeleton of the hemocytes which significantly reduce their phagocytic activity, due to the decreased adhesive ability of the hemocytes (Cima et al., 1999). Hence, the hemocyte membrane instability induced by these contaminants may cause a reduction in phagocytic activity. The change in the expression of membrane receptors (Sami et al., 1993) may interfere with the ability to recognize the non-self, which is essential for phagocytosis (Hannam et al., 2009). Phagocytosis is known to be principally dependant on cell membrane functioning, and any disruption in membrane activities modulates the process (Wootton and Pipe, 2003). Phagocytic cells normally undergo respiratory burst activity and produce more cytotoxic oxidants upon stimulation. The production of ROS and reactive nitrogen species by phagocytic cells are controlled in part by NADPH oxidase and NOS. NADPH oxidase is a plasma membrane-localized enzyme complex which facilitates electron transfers from cytosolic NADPH to molecular oxygen through an intermediary flavoprotein and cytochrome (Nappi and Ottaviani, 2000). NADPH-oxidase driven superoxide anions produce toxic metabolites which can destroy invasive pathogens and this vital cellular defense reaction is well characterized in mollusks (Adema et al., 1991; Arumugam et al., 2000). The high positive correlation observed between THC and phagocytosis clearly revealed that both markers are interdependent of stress or stimulation.

As mentioned above the generation of superoxide anions produces reactive oxygen metabolites capable of invading invasive pathogens, and in mollusks it is reported that low concentrations of contaminant exposure enhance superoxide generation (Anderson et al., 1997; Pipe et al., 1999; Dyrynda et al., 2000). However, the persistent increased levels of intra and extra cellular superoxide observed in the present study due to TBT and BaP exposure might result in tissue damage. During the recovery period extra cellular superoxide was significantly decreased in both TBT and BaP exposed abalones and the intra cellular superoxide generation showed no variation in BaP exposed groups compared with the respective control group. However, abalones previously exposed to TBT showed significant induction of superoxide during the recovery period. This suggested that TBT exposure may interact with the hemocytes of abalones, thus producing an immunotoxic effect which persists for a longer period even when there is no input of TBT. However, whether the modulation of these superoxide levels from the hemocytes of abalones exposed to both chemicals arose from increased generation or was due to the suppression of scavenging antioxidant enzymes is not known and needs to be further elucidated.

In addition, superoxide production and the increased level of NO in abalone hemocytes due to TBT and BaP exposure indicated that the hemocytes of abalone were likely to be involved in production of NO, a molecule considered as a precursor of a variety of reactive nitrogen intermediates which are synthesized from L-arginine by NOS. Although NO defense functions generated by invertebrate immunocytes have been reported, few studies have been devoted to the effects of pollutants on NO generation in mollusks (Smith et al., 2000; Kaloyianni et al., 2009). The present study showed that NO and NOS were induced by both TBT and BaP exposure, demonstrating that these two chemicals may act as immunostimulants on gastropod hemocytes. Conversely, Smith et al. (2000) report that TBT causes significant inhibition of NO and NOS in *M. edulis*. However the results from the earlier study were derived from a short term exposure to the pollutant used. Meanwhile, the same study reports that TBT induced both NO and NOS at a lower concentration (0.01 mg/L). Notably, during the recovery study, NO production in the hemocytes of TBT exposed abalones was maintained at a high level while in BaP exposed abalones it did not, which indicated a more persistent effect of TBT on abalones.

Torreilles and Guerin (1999) report that during the process of phagocytosis, cell death may increase due to peroxynitrite, an end product formed after reaction of NO with superoxides. Although the superoxide and NO production play a significant role in protecting cells from oxidant injury and scavenging radical species (Wong and Billiar, 1995; Fang, 1997), the increased levels of these free radicals may be associated with the induction of cytotoxic damage in the hemocytes of abalones exposed to TBT and BaP. However, after a 14 day recovery period abalones exposed to BaP showed no significant increase in NO and NOS compared with the respective control group. This may be due to the production of newly generated hemocytes in the animal as evidenced by the increase in THC during the recovery period. Further research is needed in order to clarify the exact mechanisms involved in the above processes.

Ordas et al. (2000) report that when the hemocytes of molluskan species are stimulated by an immunostimulant, there is an increase in oxygen consumption due to the activation of NAD(P)H-oxidase and MPO. The function of these enzymes is their involvement in the transfer of molecular oxygen to reactive intermediate species, which are highly microbicidal (Adema et al., 1991). The MPO activity was highly induced due to TBT and BaP exposure during a short exposure period (3 days), and its activity trend was similar to that observed for extracellular superoxide generation (Fig. 4). The majority of the O_2^- formed during the production of oxyradicals may be converted to the microbicidal oxidant hypochlorous acid via a series of reactions involving superoxide dismutase and MPO (Thilagam et al., 2009). After 7 days of the recovery period there was no significant change in MPO activity in abalones exposed to TBT and BaP with their respective control groups, but MPO activity decreased in the TBT-exposed abalones after 14 days of recovery. The reason for the decrease in MPO activity after a 14 day recovery period is unknown and more studies are needed to clarify the exact mechanisms involved in the above processes.

Hemocyte membrane stability assay using neutral red has been reported as an integrative biomarker in immunotoxicology. The results revealed that, under our experimental conditions, the exposure of TBT and BaP to abalones induced significant cytotoxicity in their hemocytes, as evidenced by NRR assay. Modulations in the uptake of neutral red and its retention time may reflect damage to hemocyte membrane stability. In general, the dye will be retained only in the lysosomes of healthy cells after its initial uptake and any destruction of the membrane due to contaminant will reduce the retention time. Our results showed that the hemocyte membrane stability of abalones exposed to TBT and BaP decreased as the exposure period increased. Interestingly in our study, the trend of the effects caused by BaP and TBT on membrane stability was consistent with that observed on hemocyte phagocytosis. Membrane stability is very important in the maintenance and functioning of cellular processes. The alteration of lysosomal membrane integrity may cause undesired release of hydrolases into the cytosol with consequent damage for the hemocyte cell. During the 14 day recovery period, the abalones previously exposed to BaP showed no significant change. However, the effect of TBT on the animal did not decrease even after 14 days of recovery. Similar TBT, BaP and other PAH effects on membrane stability are reported for mollusks (Matozzo et al., 2003; Wootton et al., 2003; Gopalakrishnan et al., 2009).

Lysozyme is an enzyme produced by hemocytes during phagocytosis, which actively participates in the inactivation of invading pathogens. Significant inhibition of lysozyme activity was observed when the abalones were exposed to TBT and BaP. A similar decreasing trend is reported in abalones exposed to TBT for 30 days (Zhou et al., 2010). However during recovery (7 days) the abalones previously exposed to TBT showed increase in lysozyme activity. Reduced lysozyme activity will result in lowered resistance to bacterial challenges suggesting immunosuppression was present in the host.

Loading values calculated during the PCA were used to examine the contribution of the marker to each of the components. The magnitude of the loading values for each marker is indicative of its significance to the final component which can be used to identify marker patterns. The most significant loadings to components 1 and 2 were likely to be associated with the immediate response to the contaminant, which includes NOS, membrane stability, THC, phagocytosis, NO, intra cellular superoxide generation and lysozymes. Surprisingly, MPO and the extracellular superoxide deviated relatively well from component 1 and 2. Moreover, the resulting PCA model also showed a clear trend with duration of exposure. For instance, THC, membrane stability and phagocytosis were grouped together in abalones upon BaP and TBT exposure. Nitric oxide was grouped with superoxide and NOS. Based on this result we speculated that the duration of exposure to some extent decreased the THC and reduced the membrane stability, which may have had a direct impact on phagocytosis leading to an increased production of superoxide. Moreover, from our results it was very clear that the immune system of mollusks consisted of a complex defense system with all parts being interdependent, and any impairment in immune function may be compensated for by another defense mechanism.

In conclusion, the hemocytes in invertebrates are important indicators of health and stress since they are representative cells and have a critical role in internal defense. Measurement of hemocyte function may predict the potential status of the host's health condition. Thus, it could be concluded from the present study that the exposure of abalones to TBT and BaP significantly inhibited immune related activities, and this was reflected by significant decreases in THC, membrane stability and phagocytic activity. In addition, very large amounts of free radicals were generated in the hemocytes of abalones exposed to TBT and BaP. It is commonly acknowledged that overproduction of free radicals may cause damage to cells, but whether the induction of free radicals in the abalones exposed to both chemicals in the present study would be beneficial for the host in terms of its resistance to invading pathogens remains open to further investigation. Overall, these data support the hypothesis that abalone hemocytes may represent a significant *in vivo* target of TBT and BaP toxicity. However, during recovery, most of the immune associated parameters analyzed returned to the normal level in abalones exposed to the sublethal concentrations of BaP in comparison with the respective control. This revealed that this animal could restore its immune function when the level of stress due to BaP was reduced. It was also noteworthy that, even at sublethal concentrations of TBT, most parameters tested in the TBT-exposed abalones in this study did not return to control levels during the recovery period. Observations of the difference in recovery from the toxic effect caused by sublethal concentrations of BaP and TBT indicated that the subsequent immunomodulation in abalones was dependent on the types of pollutants involved.

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