Marine Pollution Bulletin 109 (2016) 507–511

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

# Variability in antioxidant/detoxification enzymes of *Sinonovacula constricta* exposed to benzo[*a*]pyrene and *phenanthrene*

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ARTICLE INFO

*Article history:*
Received 16 March 2016
Received in revised form 3 May 2016
Accepted 4 May 2016
Available online 20 May 2016

*Keywords:*
Antioxidant responses
Biomarker
Benzo[*a*]pyrene(B(a)P)
Phenanthrene(PHE)
*Sinonovacula constricta*

ABSTRACT

The purpose of this study was to investigate the toxic effects induced by benzo[*a*]pyrene and phenanthrene. For this purpose, a study was performed on the clam exposed to 0.0, 0.5, 4.5 $\mu g L^{-1}$ B(a)P and PHE for 15 days using parameters of antioxidant defenses and oxidative stress. Antioxidant biomarkers including ethoxyresorufin-*O*-deethylase, glutathione *S*-transferase, superoxide dismutase, and glutathione and rylhydrocarbon hydroxylasein gills of *Sinonovacula constricta*, were analyzed after a 1-, 3-, 9- and 15-day exposure to seawater containing B(a)P and PHE. Integrated biomarker response was calculated by combining multiple biomarkers into a single value. The results showed that the activity of all antioxidant biomarkers was induced throughout the exposure period, and different patterns of variations were detected with exposure time. In addition, the study showed that the two concentrations used caused the activation of different general detoxification mechanisms, and the same concentration at different two PAH compounds induced different toxicity responses.



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants of petrogenic and pyrogenic origin that are widespread in marine and coastal environments (Rocha et al., 2012). Benzo[*a*]pyrene(B(a)P), a probable human carcinogen (Laffon et al., 2006) is widely used as a reference compound in studies on the toxicity of PAHs in natural communities (Aoya et al., 2003). Phenanthrene (PHE) is a priority PAH, and, although not mutagenic or carcinogenic, it is commonly used as a model substrate for studies on metabolism of carcinogenic PAHs (Zhang et al., 2014).

Antioxidant defenses are sensitive to environmental contamination and can be used as biological indicators of aquatic environmental health (Sturve et al., 2008). The defense mechanisms mainly allow metabolization, detoxification, sequestration, excretion and antioxidant protection to help the organisms to live under stress (Kaur and Kaur, 2015). Bioaccumulation of toxic substances triggers redox reactions generating free radicals that induce biochemical alterations in organism's tissues (Woo et al., 2006). B(a)P and PHE can cause oxidative stress by inducing a high level of reactive oxygen species (ROS), which could be related to cellular damage and apoptosis (Bo et al., 2014). Antioxidant enzyme systems are a well-developed regulatory mechanism scavenging ROS for protective mechanisms and avoid oxidative stress, including non-enzymatic small antioxidant molecules (such as reduced glutathione (GSH)) and a cascade of enzymes (such as superoxide dismutase (SOD) (Ren et al., 2015). Antioxidant/detoxification enzymes thus play a crucial role in maintaining organism homeostasis and their induction reflects a specific response to pollutants (Lavradas et al., 2016; Tsangaris et al., 2010; Schmidt et al., 2013). Therefore, antioxidant/detoxification enzymes have been proposed as biomarkers of contaminant-mediated oxidative stress in a vary from aquatic organisms (Regoli et al., 1998). An integrated biomarker response index (IBR) was developed to assess aquatic environmental quality based on the response from antioxidant enzymes to pollutants (Serafim et al., 2012). The IBR index can be useful for the quantitative assessment of the toxicological effects of PHE on *Morula granulate* (Bhagat et al., 2016).

Bivalve mollusks, owing to their feeding behavior, readily take up lipophilic organic contaminants, such as PAHs, from the marine environment, with a variety of physiological effects (Livingstone et al., 1995). In this study, an attempt has been made to select the biomarker enzyme for assessing B(a)P and PHE effects on biotransformation in *Sinonovacula constricta*, an Agamaki clam (an economically important shellfish in Asia). Levels of antioxidant/detoxification enzymes were estimated in gills as an indicator of the stress after 15 days exposure to B(a)P and PHE. The experiment was to ascertain the time taken for reversals of enzyme activity to normal after exposure to these two PAH. Little work has been done on the effect of PAH on antioxidant/detoxification enzymes of clams. Therefore, this work holds great importance for environmental risk assessment. A group of frequently used oxidative stress biomarkers (SOD, CST, GSH, EROD, and AHH) were selected for biochemical assays, and the integrated biomarker response (IBR) index was applied to assess the comprehensive effects of the different exposures to B(a)P and PHE.

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http://dx.doi.org/10.1016/j.marpolbul.2016.05.013
0025-326X/

## 2. Materials and methods

### 2.1. Chemicals

B(a)P (purity, 99%) and PHE (CAS# 85-01-8) were purchased from the Sigma Company. Acetone was used as the vehicle to dissolve the chemical. All other chemicals and solvents were of analytical grade.

### 2.2. Animals and treatments

Healthy *S. constricta* averaging 14.62 ± 2.26 cm in body length, were obtained from a commercial farm in Qidong, Jiangsu, China. The clams were subjected to an acclimation period of one week in plastic tanks of 100 L capacities before the exposure test. The tanks were containing aerated seawater (salinity 26‰, pH 8.1) at 23.6 ± 1.5 °C for seven days.

After acclimatization, the clams were exposed to different B(a)P concentrations (0.5 and 4.5 μg $L^{-1}$) and PHE (0.5 and 4.5 μg $L^{-1}$). There were three triplicates for each level and 60 clams in each aquarium. Seawater containing the same concentrations of B(a)P and PHE was added to maintain the corresponding concentrations of B(a)P and PHE during the experiment. B(a)P and PHE were first dissolved in acetone. The final acetone concentration was 0.001% in all tanks including the control ones (the acetone test has been done in a preliminary experiment with the result that there was no influence on clam). All water was renewed every morning, and dissolved the amounts necessary for the concentrations in the aquarium.

Clams were sampled 1, 3, 9, and 15 days after the end of the acclimatization period. The gills of each clam were used for the further assay. Twelve clams were sampled for each day and concentration, including controls.

### 2.3. Tissue sample preparation

Gill fractions were prepared according to the methods described by Bebianno and Barreira (2009). Samples were kept on ice during the whole procedure. After centrifugation for 30 min at 4 °C (3000 rpm), the supernatants were collected for analysis of the activity of SOD, EROD, GST, GSH and AHH and the protein content.

#### 2.3.1. EROD assay

EROD activity was then measured according to Pohl and Fouts (1980). Resorufin was identified and concentrations were calculated by comparison to retention times and response of resorufin standards. Blanks corresponded to $t = 0$ min and quantification was achieved with standard additions of resorufin.

#### 2.3.2. GST activity assay

GST activity was determined in the cytosolic fraction using 1-chloro-2.4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). The reaction mixture contained 200 μL supernatants, 2 mL phosphate buffer (0.125 mol $L^{-1}$, pH 7.7), 0.2 mmol $L^{-1}$ CDNB and 0.2 mmol $L^{-1}$ GSH.

#### 2.3.3. SOD activity assay

SOD was estimated by measuring an increase in absorbance at 540 nm (Kono, 1978). Twenty-five percent homogenate was prepared for 50 mM sodium carbonate buffers (pH 10.0). The reaction mixture consisted of 50 mM sodium carbonate buffers (pH 10.0), 20 mmol $L^{-1}$ hydroxylamine-HCl, 96 μM NBT and 0.6% Triton X-100.

#### 2.3.4. GSH activity assay

Glutathione (GSH) contents were measured using spectrofluorometric assay by slight modifications of the method of Hissin and Hilf (1976). GSH reacts with 5.50-dithiobis-2-nitrobenzoic acids, and the absorbance spectra of the product have a maximum absorbance at 340 nm.

#### 2.3.5. AHH activity assay

AHH activity was assayed by the method of Pan et al. (2008); dilutions were made as necessary to bring the activity within the linear range of the assay.

### 2.4. Calculation of the IBR

A method of integrating all the measured biomarker responses to one general "stress index", termed "Integrated Biomarker Response" (IBR), was applied to evaluate an integrated impact on toxicants (Beliaeff and Burgeot, 2002). A multivariate analysis was used to assess the integrated response based on biomarker data obtained from two PAH. Data were analyzed using a graphical method described in Beliaeff and Burgeot (2002) to combine the different biomarkers into an integrated biomarker response (IBR) calculated as:

$$\mathrm{IBR} = \sum_{i=1}^{n} A_i,$$

where A*i* is the area represented by biomarker *i* on a star plot graphic. The IBR calculations were performed with Excel software (Microsoft, WA, USA).

### 2.5. Statistical analyses

The results are reported as mean ± standard error (SE). Data were checked for normality variances (Shapiro–Wilk's test) and homogeneity of variances (Levene's test). Statistical analyses were carried out by one-way ANOVA using the Dunett's test to evaluate whether the means were significantly different. If an invalidation of normality and variance homogeneity was observed, data were analyzed using a non-parametric Kruskal-Wallis ANOVA on ranks, followed by a Mann-Whitney *U* pairwise comparison test. Correlation between antioxidant responses to B(a)P and PHE concentration was assessed by Pearson correlation coefficient. Using the two-tailed tested the significance. The differences were regarded as statistically significant at $p < 0.05$. The statistical computations were performed with SPSS 13.0 for Windows.

## 3. Results

### 3.1. B(a)P and PHE experiment

The effect of exposure to B(a)P on activities of antioxidant enzymes (SOD, GST, GSH, AHH and EROD) in the tissues of gills of *S. constricta* was shown in Figs. 1 and 2. During the experiment, the activities of AHH, EROD, SOD and GST in the tissues of gills increased and then came into equilibrium. GSH contents in the tissues of gills tended to alter slightly ($p > 0.05$), while a significant decrease ($p < 0.05$) occurred in the tissues of gills exposed to 4.5 μg $L^{-1}$ B(a)P and PHE after 3 days.

### 3.2. The correlation analysis with B(a)P and PHE on detoxification metabolism parameters in S. constricta tissues

As shown in Table 1, during B(a)P and PHE exposure times, the activities of GST content, EROD, GSH, SOD and AHH activities in gill showed obvious linear relationships. Among these indexes, GST content and AHH activity showed a significant and positive correlation with B(a)P and PHE concentrations. There were no correlations for B(a)P and PHE concentration with SOD activity for all experimental period. As for EROD activity showed a significant and positive correlation with B(a)P and PHE concentrations after 9 days and 15 days B(a)P and PHE exposures. While the levels of GSH activity were negatively correlated with B(a)P and PHE exposures.