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Biochemical responses in the gills of *Meretrix meretrix* after exposure to treated municipal effluent

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ABSTRACT

The biochemical effects in marine bivalves exposed to increasing concentrations of treated municipal effluent (TME), as discharged into receiving marine waters, are investigated. The effluent was collected from a municipal sewage treatment plant (STP) in Qingdao (China). *Meretrix meretrix* were exposed to effluent volume ratio (EVR, ratio of effluent volume accounted for tailwater seawater mixture) 0%, 1%, 5%, 10%, and 20% (v/v) TME for 15 days and the following biochemical responses in gills were measured: (1) the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione (GSH) content, and lipid peroxidation levels of malondialdehyde (MDA), as oxidative stress biomarkers; (2) the activity of 7-ethoxyresorufin O-deethylase (EROD) and gluthathione S-transferase (GST), as phase I and phase II conjugation enzymes, respectively; (3) acetylcholinesterase (AChE), as a biomarker of neurotoxicity, and (4) metallothioneins (MTs), as proteins strongly induced by heavy metals. Most of the biochemical indices present high and significant variation frequency (above 50%). There is enhancement in the antioxidant enzymes, EROD, GST, AChE, and MTs, as well as consumption of GSH. The current experimental results suggest that effluent with concentrations less than 20% (v/v) do not cause lipid peroxidation damage. This implies that the activated defense is sufficient to protect the bivalves' gill tissues from cytotoxicity produced by the effluent. Furthermore, GSH, GPx, MTs, and GR are suitable, and sufficiently sensitive, biomarkers to indicate the pollution levels in marine environments receiving such effluent.

1. Introduction

The inflows of sewage treatment plants (STPs) mainly contain sanitary sewage, industrial wastewater, and urban surface runoff. These inflows contain heavy metals, surfactants, some drugs, endocrine disrupting chemicals (producing harmful effects at very low concentrations), and various other compounds (e.g. chlorinated hydrocarbons and polycyclic aromatic hydrocarbons (PAHs)). Such pollutants cannot be removed effectively using existing secondary sewage treatment technology and advanced treatment technologies, e.g. high-loading activated sludge methods (Watkinson et al., 2009; Bolong et al., 2009). They are emitted with the outflow from the STP (i.e. in the effluent) and enter into the receiving water where they act as the major source of chemical pollutants in the marine environment.

In the receiving water, the concentrations of the pollutants mostly lie below the detection limit of analytical methods. However, research shows that some xenobiotic compounds can produce ecological effects at trace levels. For example, they may have acute and sub-lethal toxic effects on fishes and invertebrates exposed to the effluent even at concentrations below 1% (volume ratio) (Hannam et al., 2009; Zhu et al., 2008). The mixed system composed of trace pollutants is not capable of satisfying the evaluation needs for integration with seawater by merely using chemical analysis to detect the pollutant concentration in the sea area receiving the effluent. In recent years, biomarkers have been widely used in comprehensive monitoring projects due to their response sensitivity towards pollutants (MAP, 2005; WGBEC, 2007). Research from abroad (Veldhoen et al., 2009) has reported that using sensitive biomarkers to monitor the influence of effluent pollution is an effective and viable method. However, there has been no research concerning the use of biomarkers to monitor pollution levels in the sea areas receiving effluent in China.

In this study, *Meretrix meretrix*, a bivalve commonly found in the Qingdao sea area and widely used for monitoring of water contamination, were exposed to STP effluent at different concentrations. *M. meretrix*'s gills were subsequently analyzed for five oxidative stress markers, including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and reduced glutathione (GSH), and other substances,

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including glutathione S-transferase (GST), 7-ethoxyresorufin O-deethylase (EROD), acetylcholinesterase (AChE), metallothioneins (MTs), and malondialdehyde (MDA).

The aim of this study was to screen sensitive biomarkers which was used to provide effective biological indicators for comprehensive evaluation of the marine environment receiving the effluent, through exploring the effects and correlations of dose and exposure time on the biochemical levels.

2. Materials and methods

2.1. Materials

Treated municipal effluent (TME), which would be directly discharged into receiving marine water, was collected from a sewage treatment plant (Qingdao, China). Four effluent samples collected over 24 h were mixed in equal volumes and transferred to the laboratory, kept at 4 °C and used the next day.

A collection of *M. meretrix* of similar size were gathered from the culture zone of the Daguandao sea in the Laoshan Bay area (Qingdao, China) and cleaned in natural sea water at 15 ± 1 °C for 7 days (pH of 7.90 ± 0.02, salinity of 32‰). The mussels were fed with suspensions of the green alga *Chlorella pacifica* (1.3×10^7 cells per liter per day) during this pre-culture process. An air pump was used for continuous aeration to maintain a constant level of dissolved oxygen (6 ± 0.5 mg L^{-1}).

2.2. Effluent exposure assay

For the exposure experiments, clams were divided into groups of 90 per glass cylinder (10 L) and then treated using 0%, 1%, 5%, 10%, and 20% (v/v) of TME for 15 days and fed with suspensions of the green alga *Chlorella pacifica* (1.3×10^7 cells per liter per day). Three glass cylinders of 90 clams were adopted per treatment. During the treatment period, the clean natural seawater and TME were changed daily in each glass cylinder. The clams' gill tissues were collected after different periods of time (0, 3, 6, 9, 12, and 15 days) and stored at −80 °C.

2.3. Sample pre-treatment and analysis of biochemical parameters

2.3.1. Preparation of tissue extract

Gill samples from each exposure treatment were divided into two aliquots. For measurement of protein content and most biomarkers except MTs, the sample was homogenized (1:4, w/v) with cold Tris–HCl buffer (0.02 M, pH 7.8). The homogenate was centrifuged (10,000*g*, for 15 min at 4 °C) and the supernatant carefully collected. For detection of MTs, the sample was homogenized (1:4, w/v) with cold Tris–HCl buffer (0.02 M, pH 8.6) containing 0.5 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride as an antiproteolytic agent, and 0.01% β-mercaptoethanol as a reducing agent. The homogenate was centrifuged (25,000*g*, for 20 min at 4 °C) and the supernatant used for MT quantification.

2.3.2. SOD, CAT, GR, GPx, MDA, and protein assays

The activities of these biomarkers and the protein content were detected using a commercially-available assay kit (Nanjing Jiancheng Bioengineering Institute, China). The resulting data was expressed in units of U mg^{-1} protein, U g^{-1} protein, U g^{-1} protein, U mg^{-1} protein, nmol mg^{-1} protein, and g L^{-1}, respectively.

2.3.3. AChE assay

AChE activity was determined according to the method described by Ellman et al. (1961). The method is based on a coupled enzyme reaction involving acetylthiocholine as the specific substrate for AChE and 5,5′-dithio-bis-2-nitrobenzoate (DTNB) as an indicator for the enzyme reaction at 412 nm. Results are expressed as nmoles of thiocholine produced per minute per milligram protein (nmol min^{-1} mg^{-1} protein).

2.3.4. GSH assay

GSH content was determined in alkaline medium by measuring the intensity of the fluorescence of the compounds produced by the conjugation of o-phthalaldehyde and GSH using excitation/emission wavelengths of 350 nm/430 nm (Zhang et al., 1993). The results were expressed in μmoles of GSH per gram protein (μ mol g^{-1} protein).

2.3.5. GST assay

GST activity was measured according to the method of Habig and Jakoby (1981) using 1-chloro-2, 4-dinitrobenzene and GSH as substrates. Absorbance was measured at 340 nm, and activities were expressed as nmoles of conjugated product formed per minute and per milligram of protein (nmol min^{-1} mg^{-1} protein).

2.3.6. EROD assay

EROD was tested using a commercial kit (America Genmed Gene Company). The activity of EROD was measured by checking the formation of resorufin using spectrofluorometry (550 nm excitation, 580 nm emission wavelengths). EROD activity was expressed as pmol min^{-1} mg^{-1} protein.

2.3.7. MT assay

MT content was evaluated using the spectrophotometric method of Viarengo et al. (1997). Three volumes of absolute ethanol (−20 °C) were added to the supernatant resulting from ethanol/chloroform extraction to precipitate the MTs. The MT pellets were resuspended in NaCl/HCl/EDTA to remove metal cations still bound to the MTs. After this Ellman reagent (pH 8.0 phosphate buffer containing DTNB) was added to the solution. The DTNB reacts with the thiol (–SH) groups in the MTs. Quantification of the MTs was performed spectrophotometrically at 412 nm. Standard solutions of reduced GSH (0–400 μM) were used for calibration and the data expressed as μmoles of –SH g^{-1} protein.

2.4. Data analysis

The results were expressed as mean ± standard deviation (SD; $n=3$). SPSS software (version 17.0) was used for the statistical analysis. First, the homogeneity of the variance of the parameters was judged using *F*-tests and Levene tests. If the variances were found to be homogeneous, then the differences between the parameters were compared for multiple times using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Otherwise, a Games–Howell test method was employed for difference examination. Finally, the correlation between dose and effect were investigated using a bivariate Pearson correlation method. The statistical significance levels were set at $P<0.05$ and $P<0.01$.

3. Results

3.1. Change in activity of the antioxidant enzymes

The changes in activity of the four antioxidant enzymes (SOD, CAT, GPx, and GR) and the MDA content present in the gills of *M. meretrix* as a function of EVR and exposure duration are shown in Fig. 1. Before the experiment, the activities of SOD, CAT, GPx, and GR were 95.54 U mg^{-1} protein, 8.97 U g^{-1} protein, 11.09 U mg^{-1} protein, and 2.62 U g^{-1} protein, respectively. During culturing, the activity of these antioxidant enzymes in the control group showed little variation.