RESEARCH REPORT

Mercury exposure modulates antioxidant enzymes in gill tissue and hemocytes of *Venerupis philippinarum*

X Chen[1], R Zhang[1], C Li[1], Y Bao[2]

[1]School of Marine Sciences, Ningbo University, Ningbo, Zhejiang Province 315211, PR China
[2]Zhejiang Key Laboratory of Aquatic Germplasm Resources, Zhejiang Wanli University, Ningbo 315100, PR China

Abstract

Mercury (Hg), one of the most hazardous and persistent contaminants, is widespread in the aquatic environment. To establish an effective Hg monitoring strategy, the mRNA expression profiles of four antioxidant enzymes, as well as SOD enzymatic activities and MDA content were investigated at two sublethal $HgCl_2$ exposure doses of 5 and 50 µg L^{-1} in *Venerupis philippinarum* gill tissue and hemocytes at 1, 2, 4 and 5 days. All parameters tested showed significant differences between the experimental and control groups at the various time points with tissue-specific manners. GST displayed a dose-dependent increase expression profiles in the two examined tissues. Also decrease expression patterns were detected in Trx and CYP414A1 in both gill and haemocytes with a significant positive relationship of 0.915 in the form tissue ($p < 0.01$). A positive relationship was found in those of SOD expression and the SOD enzymatic activities in hemocytes (0.683, $p < 0.05$). Taken together, we found that gill tissue is more suitable for biomarker identification compared to that of hemocytes because of lower variation. This study provides new evidences that mRNA expression of Trx, CYP414A1 and GST in gill tissue has a strong potential as a biomarker for marine mercury monitoring.

Key Words: *Venerupis philippinarum*; mercury; gene expression; enzymatic activity

Introduction

The Manila clam *Venerupis philippinarum* is a major aquaculture species worldwide and is widely distributed in high densities in coastal intertidal areas. Due to its wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection, and high bioaccumulation of heavy metals (Laing and Child, 1996; Kim *et al.*, 2001; Baudrimont *et al.*, 2005; Liu *et al.*, 2011a, b ,c; Wu *et al.*, 2011), the Manila clam exhibits most of the criteria that define a bio-indicator for metal pollution monitoring and has therefore been considered a useful bio-indicator in marine biology and ecotoxicology (Liang *et al.*, 2004; Ji *et al.*, 2006; Zhang *et al.*, 2011a, b, c; Liu *et al.*, 2013a, b). Recent toxicological studies have elucidated distinct biochemical and genetic responses of *V. philippinarum* to heavy metals and other toxic contaminants, further supporting the proposition that *V. philippinarum* may be useful for monitoring marine and coastal pollution (Blasco and Puppo, 1999; Ji *et al.*, 2013; Nasci *et al.*, 2000; Moraga *et al.*, 2002; Irato *et al.*, 2003; Wu *et al.*, 2013a, b ,c; Zhang *et al.*, 2011d, 2012a).

Mercury (Hg) is a widely distributed pollutant and has been listed as a high priority pollutant by many international agencies because of its persistence in environments and high toxicity to organisms (Jiang *et al.*, 2006). Increasing evidences have demonstrated that the mechanism underlying mercury toxicity is closely related to the generation of reactive oxygen species (ROS) and the perturbation of antioxidative defense systems (Larose *et al.*, 2008; Liu *et al.*, 2011a). Organisms can hold in check and detoxify the production of ROS with antioxidative defense systems comprising of a wide variety of enzymatic and non-enzymatic molecules to balance the harmful effect of ROS, which include superoxide dismutase (SOD), glutathione-S-transferase (GST), cytochrome P450s (CYPs) as well as non-enzymatic molecule of thioredoxins (Trxs).

SODs are metalloenzymes that represent one of the important lines of defense against ROS, particularly against the superoxide anion (Wang *et*

Corresponding authors:
Chenghua Li
Ningbo University
818 Fenghua Road
Ningbo, Zhejiang Province 315211, PR China
E-mail: lichenghua@nbu.edu.cn
Yongbo Bao
Zhejiang Wanli University
8 South Qianhu Road
Ningbo, Zhejiang 315100, PR China
E-mail: bobbao2001@gmail.com

al., 2013). SODs can catalyze the dismutation of superoxide into molecular oxygen and hydrogen peroxide (Fridovich, 1995). GSTs are a superfamily of multifunctional phase II enzymes primarily involved in the detoxification of both endogenous and exogenous electrophiles (Zhang et al., 2012b). Moreover, GSTs have been found to play a critical role in mitigating oxidative stress in all life forms (Lee et al., 2008), and GST activities have been widely used as potential biomarkers for monitoring environmental pollution (Hoarau et al., 2002; Shailaja and D'Silva, 2003; Cunha et al., 2007). Trxs are members of an evolutionarily conserved family of redox-active proteins and function to scavenge reactive oxygen species (ROS) (Wang et al., 2011). Cytochrome P450s (CYPs) comprise one of the largest and most versatile heme-thiolate protein families. They can catalyze the oxidation of a wide variety of exogenous compounds or xenobiotics. CYP414A1 is classified as a member of a new subfamily of cytochrome P450 (Zhang et al., 2012c). CYP414A1 has been shown to respond to various xenobiotic stresses and could potentially serve as a candidate biomarker of heavy metals. The lipid peroxidation product malonyldialdehyde (MDA) has also been widely utilized as an indicator of oxidative status (Roméo and Gnassia-Barelli, 1997).

To establish a rapid and accurate detection system for Hg in marine environments, specific biomarkers are the first priority. As filter-feeding organisms, molluscs expose to various environment pollutants including heavy metals in the aquatic environment. Gills of the bivalves remain in direct interaction with the surrounding aquatic environment and their biochemical profiles reflect the adverse environmental impact on the animal (Gregory et al., 2002) and the study of biochemical biomarkers in this organ is usually employed in environmental monitoring studies (Medeiros et al., 2008; Zanette et al., 2008). Meanwhile, hemocytes of molluscs are the first line of defense against environmental exposure, through phagocytosis and stimulation of respiratory burst and widely used as models in environmental toxicology, in order to determine the target of heavy metals' toxic effects (Bouki et al., 2013). In the present study, the expression levels of four antioxidant enzymes and malondialdehyde (MDA) contents were assayed in Venerupis philippinarum gill and hemocytes following two

doses of Hg exposure. Their correlations were further analyzed to select reliable multi-indicators for Hg biomonitoring.

Materials and Methods

Experimental clams, Hg exposure, and tissue collection

Venerupis philippinarum (wet weight 8.65 ± 1.24 g) were purchased from Ningbo, Zhejiang Province, China and acclimated for three days prior to the experiment. The temperature of the aerated artificial seawater (salinity 25 psu) was maintained at 18 - 20 °C throughout the experiment. In the challenge experiment, approximately 450 clams were randomly divided into three tanks (90 cm×60 cm×40 cm), each containing 150 individuals in 30 L seawater. The clams in the two treatment tanks were exposed to different concentrations of $HgCl_2$, either 5 or 50 µg L^{-1}. The low experimental concentration (5 µg L^{-1}) of Hg^{2+} can be found in heavily polluted seawaters along China coast (Zhang, 2001). The untreated individuals were selected as a control group. The hemolymphs from the control and challenge groups were collected using a sterile 5 ml syringe containing 1 ml of Alseve buffer (ALS buffer: 60 mM glucose, 27.2 mM sodium citrate tribasic, 9 mM EDTA, 385 mM NaCl, pH 7 and 1000 mOsm/l), then centrifuged at 800 rpm for 5 min to harvest the hemocytes. Gill were were sheared using sterilized scissors and tweezers, mixed together, and then ground into a fine powder using liquid nitrogen at 0, 1, 2, 4 and 5 days following $HgCl_2$ exposure. All the samples were then stored at −80 °C for RNA or protein extraction. We performed five replicates for each experimental groups as well as for the control group.

Temporal expression profiles of candidate genes under $HgCl_2$ exposure

Total RNA was isolated from clam hemocytes or gills using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using the MMLV First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Bio Basic Inc.). The expression levels of SOD, GST, Trx and CYP414A1 were measured by qPCR. The primers used for qPCR are shown in Table 1. Two clam β-actin primers were used to amplify a 121 bp fragment as an

Table 1 Primers used in the present study

Primer	Sequence (5'—3')	Product size	Gene ID
β-actin (forward) β-actin (reverse)	CTCCCTTGAGAAGAGCTACGA GATACCAGCAGATTCCATACCC	121bp	EF520696.1
SOD (forward) SOD (reverse)	GTGCAGGTCCTCACTATAACCCA GACAACTCGTGACCACCTTTACC	226 bp	GQ384412
GST (forward) GST (reverse)	CATTGCCCGTGCTTACTATTGAC TGTTCCTTTCTTCCTCGTTATCC	207 bp	GQ384392
TRx (forward) TRx (reverse)	GGACGTTGATGATGTTTCGGAGGT TTTCCAGTTCATCAGCATCAGCCC	125bp	JF499393
CYP414A1 (forward) CYP414A1 (reverse)	AGGACCGAGGTCATGTTTAG GGATTTAGAGTTGTCGCCAG	147bp	HQ234335
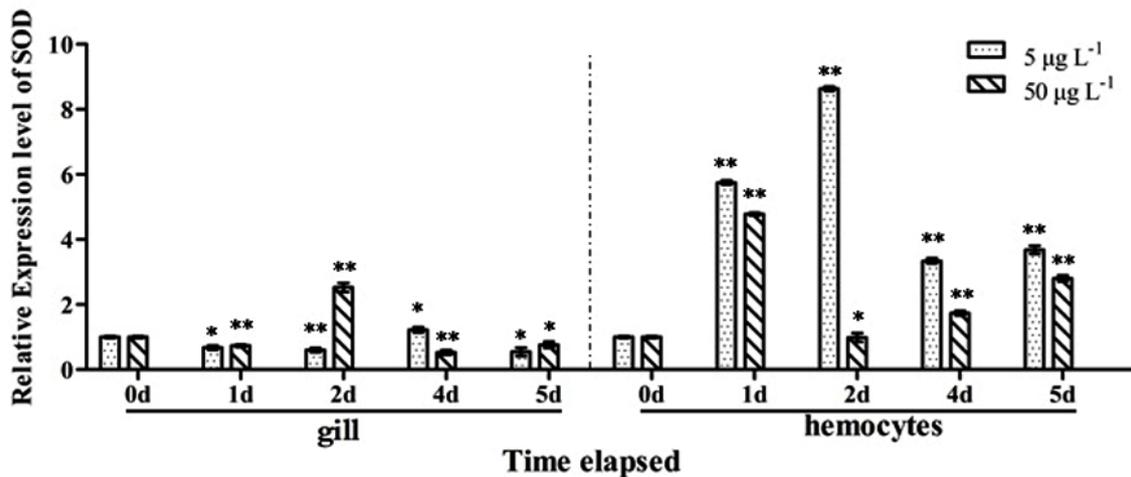
Fig. 1 Time course expression of SOD in gill tissue and hemocytes of *V. Philippinarum* after exposure to different doses of $HgCl_2$ as measured by qPCR at 0, 1, 2, 4 and 5 days.

internal control to verify successful reverse transcription and to calibrate the cDNA template. qPCR amplification was performed using a Rotor-Gene 6000 real-time PCR detection system. Real-time PCR amplifications were performed in a total volume of 20 μL containing 10 μL of 2×SYBR Green Mix (Takara), 4 μL of the 1:20 diluted cDNA, 1 μL of each primer (10 mM) and 4 μL of PCR-grade water. The qPCR parameters included a denaturing step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. Melting analysis of the amplified products was performed at the end of each PCR to confirm that a single PCR product was generated. The $2^{-\Delta\Delta CT}$ method was employed to analyze the expression level of candidate genes (Li *et al.*, 2011). The values obtained denoted the n-fold difference relative to the calibrator. The data are presented as relative mRNA expression levels (means ± SD, *n* = 5).

SOD activity assay
SOD activities were determined by the method of inhibition of tetrazolium salt WST-1 reduction to formazan with xantine/xantine oxidase used as a superoxide generator. Briefly, 0.3 g of gill tissue was homogenized in liquid nitrogen and resuspended in 3 mL normal saline. Hemocytes from 18 mL of hemolymphs were used for protein extraction. For the enzymatic assay, the supernatant was collected by centrifuging at 3,000 rpm for 10 min. Protein concentration was quantified following Bradford (1976). Absorbance was monitored at 550 nm. The total SOD activity was expressed in U/mg of protein, with one unit of SOD being defined as the amount of sample that produced 45 to 50 % inhibition under the assay conditions.

MDA content assay
MDA content was measured by the formation of thiobarbituric acid (TBA) reactive substances (TBARS) using commercial kits (Jiancheng Bioengineering Institute, China). Briefly, the reaction mixture containing 0.2 mL tissue homogenate, 0.2

mL 8.1 % SDS, 1.5 mL 20 % acetic acid buffer (pH 3.5), 1.5 mL 1 % TBA and 1 mL distilled water was heated at 95 for 60 min in a water-bath, then cooled and centrifuged at 3000 rpm for 15 min. The absorbent value was assayed at 532 nm to calculate the content of MDA according to the following formula:

$$MDA content = \frac{OD_M - OD_C}{OD_S - OD_C} \times S \div P$$

In the formula, OD_M is the OD value of the measured sample, OD_S indicates the OD value of the standard sample in the kit, OD_C is the OD value of the control, S is the concentration of the standard sample (10 nmol/ml) and P is the protein content (mg/g tissues).

Statistical analysis
All data were subjected to one-way analysis of variance (ANOVA) followed by the multiple Duncan test to test for differences between the treatment and control groups at each sampling time. Significant differences between the treatment group and the corresponding control group at each time point are indicated with one asterisk for *p* < 0.05 and two asterisks for *p* < 0.01. Spearman correlation analysis was conducted with SPSS 19.0 for Windows (SPSS). Significant differences between the treatment group and the corresponding control group at each time point are indicated with one asterisk for *p* < 0.05 and two asterisks for *p* < 0.01.

Results and Discussion

Expression profiles of SOD in gill tissue and hemocytes at the mRNA and protein levels
The mRNA relative expression of SOD in gill tissue and hemocytes upon $HgCl_2$ exposure are shown in Figure 1 and SOD activities are indicated in Figure 2. Divergent expression trends of SOD mRNAs

300
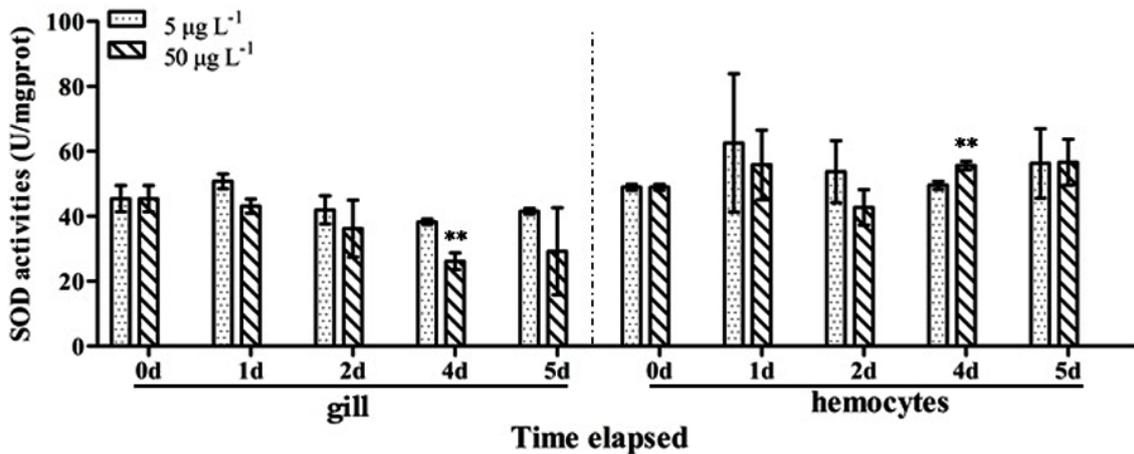
Fig. 2 SOD activities in gill tissue and hemocytes of *V. philippinarum* exposed to $HgCl_2$ (5 µg L^{-1}, 50 µg L^{-1}) for 0, 1, 2, 4 and 5 days.

were detected not only in different tissues but also under different exposure doses. These results are consistent with the the the fact that higher variation expression profiles of metalloenzymes like SODs (Kim *et al.*, 2007). At a concentration of 5 µg L^{-1} Hg exposure, the expression level of SOD in hemocytes increased during the first 2 d and then decreased gradually until 5 d. In contrast, the expression profiles of SOD in gill tissue decreased at the first stage and then sharply increased to its peak expression level at 4 d with a 1.22-fold increase over that of the control group ($p < 0.05$). When Hg concentration increased to 50 µg L^{-1}, the induced SOD expression occurred earlier compared to that in the lower doses group. The peak expression of SOD was detected at 2 d with a 2.46-fold increase in gill tissue ($p < 0.01$) and a 4.78-fold increase at 1 d in hemocytes. Regarding to SOD activities, Hg treatment resulted in a significant decrease SOD protein activities in gill tissue and a slight increase in hemocytes (Fig. 2). An unpaired, two-tailed *t*-test between the control and treatment groups showed there is a significant difference in SOD activity at 4 d ($p < 0.01$) following the higher level of Hg exposure. However, no significant differences were observed at other time points.

It was well-documented that Hg could induce oxidative stress in organisms by generating ROS via the mitochondrial electron transport chain (Lund *et al.*, 1991; Fang *et al.*, 2013), and the change in superoxide radical content was correlated with the induction or depression of SOD. SODs represent the first line of defense in biological systems against oxidative stress caused by excessive reactive oxygen species (ROS), in particular superoxide anion. Many researchers had analyzed SOD activity under Hg stress in bivalves other than *V. philippinarum*. Verlecar *et al.* (2008) reported inhibited SOD activity at day 5 and increased SOD activity at day 15 in the digestive gland of the mussel *Perna viridis* after exposure to 45 µg L^{-1} Hg. Exposure of the mussel *Dreissena polymorphato* to 40 µg L^{-1} Hg for 5 days resulted in an increase in

SOD activity in the digestive gland (Faria *et al.*, 2009). However, Company *et al.* (2004) reported that SOD activity remained unchanged in the gill of the mussel *Bathymodiolus azoricus* exposed to 20 µg L^{-1} Hg for 24 h. In general, the response of SOD activity to Hg exposure differs depending on the tissue, concentration, and exposure time. In the present study, the fluctuation of SOD mRNA expression and the alteration of SOD activity together indicate that SOD was involved in countering the oxidative stress caused by Hg exposure. A positive relationship between SOD mRNA expression and SOD activity was detected only in hemocytes ($p < 0.05$) (Table 2). This discrepant finding might be attributed to the existence of different isoforms of SOD in clam. mRNA expression was quantified as one of isoform types of SODs, whereas SOD activity was quantified in terms of total SOD activity (Fang *et al.*, 2013). The increased SOD mRNA expression in hemocytes at low concentration illustrates the fast adaptive response of SOD to Hg stress (Fang *et al.*, 2012). The decline of SOD mRNA expression and SOD activity might be ascribed to the stimulation of other antioxidants to defend against ROS toxicity, as suggested by Verlecar *et al.* (2008) and Jo *et al.* (2008). The discrepancies in the responses of different antioxidants are common among other researches, and several observations also highlighted that these defenses do not vary in a synchronous way, and have different time-courses of activation both at the transcriptional and translational levels (Frenzilli *et al.*, 2004; Regoli *et al.*, 2011; Giuliani *et al.*, 2013). These results suggest that, in addition to being influenced by enzymatic activity, SOD can be regulated by Hg at the molecular level in *V. Philippinarum*. Chatziargyriou and Dailianis (2010) reported that the inhibition of Se-GPx activity by Hg could promote a shift in the balance between oxidants and antioxidants in favor of oxidants, resulted in the enhancement of Hg induced oxidative and genotoxic effects. On the other hand, increased enzymatic activity and a

Table 2 Determination of correlation coefficients among the different examined parameters

	Gill						Hemocytes					
	SOD level	SOD activities	GST level	Trx level	CYP414 A1 level	MDA content	SOD level	SOD activities	GST level	Trx level	CYP414 A1 level	MDA content
SOD level	1						1					
SOD activities	0.085	1					0.683*	1				
GST level	0.573	-0.061	1				0.695*	0.4	1			
Trx level	0.427	0.317	0.378	1			0.817**	0.354	0.609	1		
CYP414 A1 level	0.573	0.427	0.561	0.915**	1		-0.329	-0.476	0.277	0.024	1	
MDA content	0.5	0.207	0.183	0.171	0.39	1	-0.622	-0.22	-0.006	-0.5	0.659*	1

*Statistical significance was denoted by $p < 0.05$, ** statistical significance was denoted by $p < 0.01$ versus the respective control group

significant attenuation of Hg toxicity were measured in Se-treated cells exposed to Hg in all cases. It's helpful to reveal SOD's ability to attenuate Hg-mediated ROS production, as well as the role of Se-GPx.

GST expression patterns following Hg^{2+} exposure

GSTs play a critical role in mitigating oxidative stress in all life forms (Hoarau *et al.*, 2002), and GST activity has been widely used as a potential biomarker for monitoring environmental pollution (Umasuthan *et al.*, 2012). In the present study, similar expression trends of GST were detected not only in different tissues but also at different exposure doses (Fig. 3). The gill GST transcripts was induced with a dose-dependent and time-dependent manners and reached its maximum level at 50 µg L^{-1} exposure dose with a 2.45-fold increase. In the hemocytes, the peak expression level was detected at 1 d with a 2.12-fold increase in the higher exposure dose group.

Many studies had indicated that metals/metalloids such as Hg were potent inductors of sulfur metabolism through an increase in GSH synthesis (Sobrino-Plata *et al.*, 2009). In addition, the level of intracellular GSH was directly proportional to GST activity, which leaded to an increase in the expression level of GST (Nugroho and Frank, 2012). In the freshwater prawn *Acrobrachium malcolmsonhii*, GSH content was found to be higher in the tissues of test prawns, suggesting the formation of glutathione conjugate to eliminate Hg in test prawns (Yamuna *et al.*, 2012).

Oxidative stress from Hg exposure was also considered to elicit antioxidant gene expression, such as expression in the GSH S-transferase system. In our previous studies, GSTs have shown high elevations in mRNA levels under heavy metal stress, thus serving as useful biomarkers (Rhee *et al.*, 2007).

Trx expression patterns following Hg^{2+} exposure

Thioredoxin (Trx) proteins perform important biological functions in cells by changing the redox state of proteins via dithiol disulfide exchange. The expression level of Trx following Hg^{2+} exposure is shown in Figure 4. At 5 µg L^{-1} Hg^{2+}, Trx in gill tissue was constantly down-regulated and reached its minimal level at 5 d with a 0.2-fold decrease compared to the untreated group ($p < 0.01$). However, a very different expression profile was detected in hemocytes at this lower exposure dose. Trx mRNA levels increased sharply to a peak at 2 d with a 12.0-fold increase, and then recovered to the original level at 5 d. At the higher concentration of 50 µg L^{-1}, an increased expression pattern was detected in gill tissue with a 3.72-fold increase relative to the untreated group at 2 d. Surprisingly, no significantly change was detected in hemocytes at this exposure dose.

As a molecule involved into counteracting oxidative stress and scavenging ROS, Trx serves as an electron donor for some peroxidases such as peroxiredoxins and is reduced by thioredoxin reductase to the reduced dithiol state (Gonzalez *et al.*, 2010). Stadtman (1993) indicated that heavy
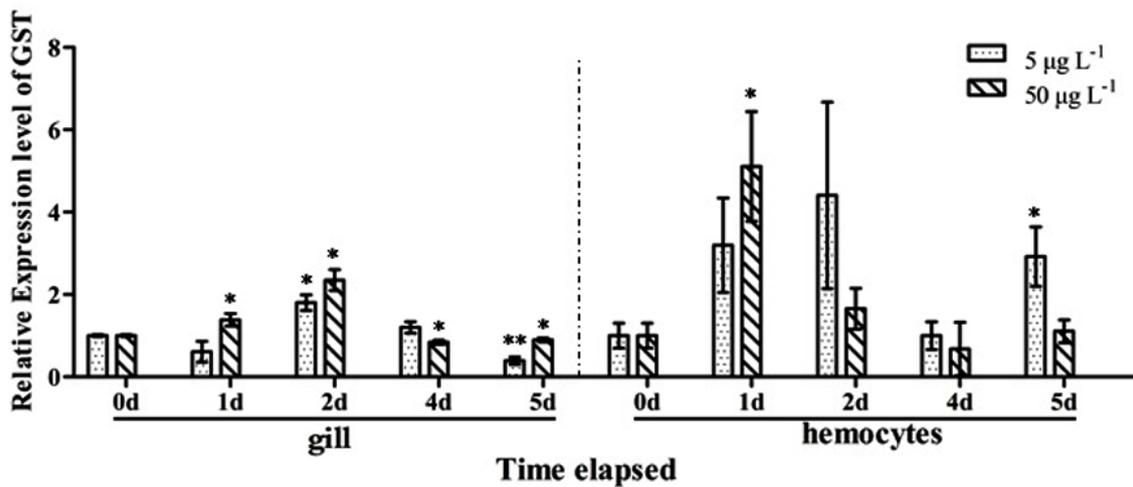
Fig. 3 Time course expression of GST in gill tissue and hemocytes of *V. Philippinarum* after exposure to different doses of HgCl$_2$ as measured by qPCR at 0, 1, 2, 4 and 5 days.

metals participated in a metal-catalyzed Fenton type reaction with superoxide or peroxide molecules to generate highly toxic hydroxyl radicals. Thus, the observed induction of Trx in the first stage could be interpreted as a response to this oxidative stress. A similar experiment by Wang *et al.* (2011) indicated that Trx was also significantly induced by 40 µg L^{-1} Cd exposure at 96 h. It has been demonstrated that Trx oxidation was significantly correlated with the inhibition of total TrxR activity during heavy metal treatment (Myers, 2012); therefore, TrxR activity should be investigated in our future work.

CYP414A1 expression patterns following Hg^{2+} exposure

Previous studies had provided evidence that Hg^{2+} modulated the expression of cytochrome P450

1A1 (CYP1A1) by affecting its transcriptional and posttranslational levels (Amara *et al.*, 2010). In the present study, the expression level of CYP414A1 is shown in Figure 5 and exhibits a dose-dependent expression profile. At the lower level of HgCl$_2$ exposure, the decreased expression profiles were both identified in the two examined tissue with different amplitudes. The minimal expression levels were detected at 5 d, with a 0.22-fold decrease in gill tissue and a 0.46-fold decrease in hemocytes. When Hg^{2+} concentration increased to 50 µg L^{-1}, we found that the expression of CYP414A1 was both elevated sharply to its peak levels following 1 d of heavy metal exposure in the two tissue. Afterwards, the expression of CYP414A1s was both down-regulated slightly, and minimal expression levels were detected at 4 d.
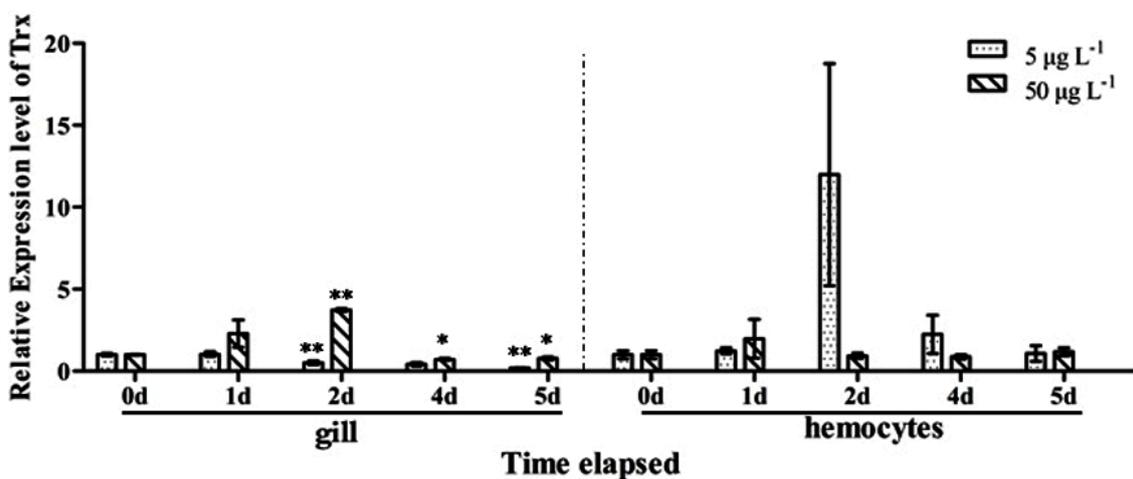
Fig. 4 Time course expression of Trx in gill tissue and hemocytes of *V. Philippinarum* after exposure to different doses of HgCl$_2$ as measured by qPCR at 0, 1, 2, 4 and 5 days.
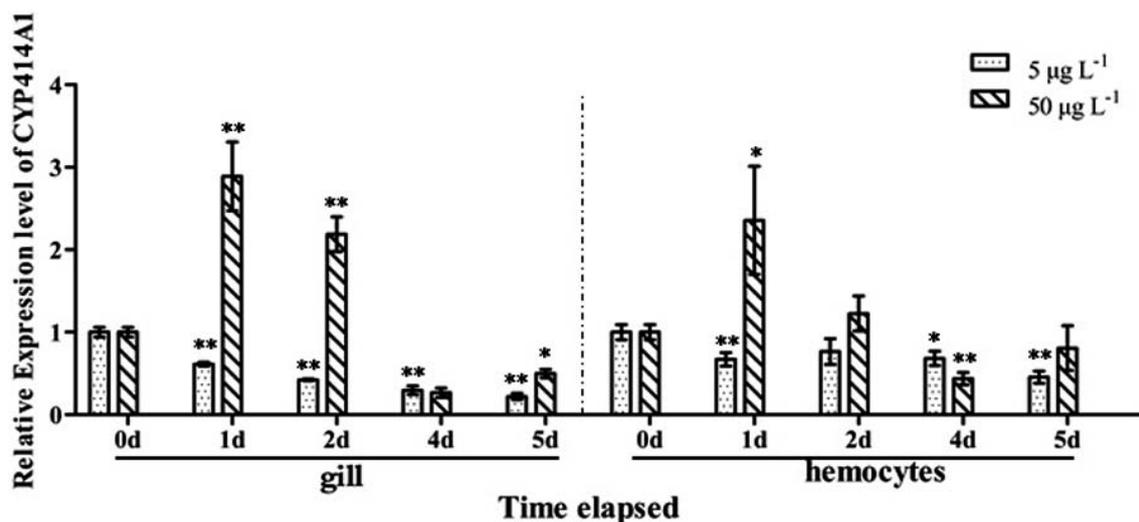
Fig. 5 Time course expression of CYP414A1 in gill tissue and hemocytes of *V. Philippinarum* after exposure to different doses of HgCl₂ as measured by qPCR at 0, 1, 2, 4 and 5 days.

Activation of the NF-κB and AP-1 (Activator Protein-1) signaling pathways had been demonstrated to be directly involved in the induction of CYP1A by Hg stimulation (Korashy *et al.*, 2008). Although the expression level of AP-1 was not investigated in that study, the induction of AP-1 and CYP414A1 was detected by our lab in the same species following di(2-ethylhexyl) phthalate (DEHP) exposure (Lu *et al.*, 2013). An analogous study revealed that the expression of CYP414A1 was significantly up-regulated by Cd exposure but sharply down-regulated by Cu exposure (Zhang *et al.*, 2012c). A possible explanation for the distinct expression profiles following differential Hg exposure doses is that no acute toxic effects were reported at the administered low dose. However, sublethal effects are subtler and take more time to become evident. Higher doses of Hg could potentially increase the toxic burden, with sub-lethal effects seen more rapidly (Amachree *et al.*, 2014).

MDA content analysis

Lipid peroxidation was a main indicator of oxidative damage, and MDA was considered to be an ideal biomarker for this process. MDA levels following Hg²⁺ exposure were investigated in our study, and the results are shown in Figure 6. In Hg-treated gill tissue, MDA content did not change significantly at the two different exposure doses. However, related to the control group, MDA content in the hemocytes decreased significantly at 4 d at the higher Hg concentration and at 5 d at the lower Hg concentration. MDA content of hemocytes after Hg treatment was 46.48 % and 45.56 % lower at the higher and lower Hg concentrations compared to the control group, respectively. These results demonstrated that Hg exposure modified the production of MDA in clams.

Most previous studies in different species had revealed significant increases in MDA content following exposure to environmental pollutants (Prakash *et al.*, 1995; Giguere *et al.*, 2003; Charissou *et al.*, 2004), implying that higher oxidative stress was produced during that period. Tavazzi *et al.*, (2000) reported that lipid peroxidation of human erythrocytes also is deeply affected by oxidative stress. However, a negative relationship between Hg exposure and MDA activity was found in our study. We speculated that this result might be due to the following reasons. Hg had a high affinity for GSH, which was the primary intracellular antioxidant agent and could bind to, and cause the irreversible excretion of, GSH, leading to the depletion of GSH and an increase in MDA (Quig, 1998). However, in the hemocytes of clams exposed to Hg, a decrease in MDA levels was associated with an increase in SOD levels. This decrease could result in an intensification of antioxidant systems, including SOD, limiting MDA formation. It was well known that MeHg was the most toxic form of Hg in the environment, which was converted by aquatic microorganisms (Compeau and Bartha, 1985). This process could not be achieved under our lab conditions, leading to a reduction in the strength of the toxic effect and minimal ROS production. In contrast, the induction of antioxidant enzymes (SOD, CYP414A1 and GST) and the non-enzyme member (Trx) was capable of suppressing this lower ROS level at an early stage, preventing oxidative stress-induced damage to lipids. Furthermore, Zhang *et al.* (2014) reported that the decrease in MDA might result from an intensification in production by the antioxidant systems, an interpretation consistent with our results. The depletion of MDA levels might also be related to the faster transformation of MDA into other metabolites (Giarratano *et al.,* 2010). Further work should be conducted to investigate the changes in ROS levels before and after Hg exposure to better understand these mechanisms.
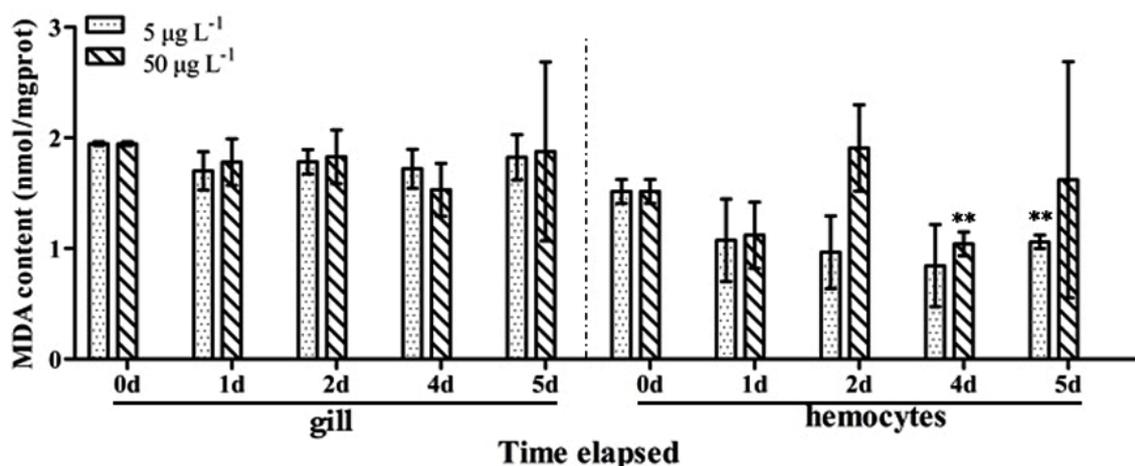
Fig. 6 MDA content of gill tissue and hemocytes of *V. philippinarum* exposed to HgCl$_2$ (5 µg L^{-1}, 50 µg L^{-1}) for 0, 1, 2, 4 and 5 days.

Relationships between examined parameters

To establish multiple biomarker detection system and improve the accuracy of detection technique, correlations analysis of these candidate biomarkers both in gill and hemocytes are further assessed and the result is showed in Table 2. There is a significant positive relationship between the expression levels of Trx and CYP414A1 expression in gill (0.915, $p < 0.01$) and Trx and SOD expression in hemocyte (0.817, $p < 0.01$). Multiple factors correlation analysis also indicate a higher consistent expression pattern among SOD, GST, CYP414A1 and MDA content ($p < 0.05$). No statistically difference are detected among other examine parameters.

Conclusions

Our work presented here addressed that mRNA expression of Trx, CYP414A1 and GST in gill tissue has a strong potential as biomarkers for marine mercury monitoring. This findings supported that gills was promising tissue for further biomarker identification. More importantly, multiple biomarker detection system sounded to be promising choice in order to improve the accuracy of detection technique. The changes of ROS levels before and after Hg exposure should be further investigated to fully understand the interaction between ROS production and antioxidant enzymes.

References

Amachree D, Moody AJ, Handy RD. Comparison of intermittent and continuous exposures to inorganic mercury in the mussel, *Mytilus edulis*: Accumulation and sub-lethal physiological effects. Ecotox. Environ. Safe. 109: 133-142, 2014.

Amara IEA Anwar-Mohamed A El-Kadi AOS. Mercury modulates the CYP1A1 at transcriptional and posttranslational levels in human hepatoma HepG2 cells. Toxicol. Lett. 199: 225-233, 2010.

Baudrimont M, Schafer J, Marie V, Maury-Brachet R, Bossy C, Boudou A, et al. Geochemical Survey and Metal Bioaccumulation of Three Bivalve Species (*Crassostrea gigas*, *Cerastoderma edule* and *Ruditapes philippinarum*) in the Nord Medoc Salt Marshes (Gironde Estuary, France). Sci. Total Environ. 337: 265-280, 2005.

Blasco J, Puppo J. Effect of Heavy Metals (Cu, Cd and Pb) on Aspartate and Alanine Aminotransferase in *Ruditapes philippinarum* (Mollusca: Bivalvia). Comp. Biochem. Physiol. 122C: 253-263, 1999.

Bouki E, Dimitriadis VK, Kaloyianni M, Dailianis S. Antioxidant and pro-oxidant challenge of tannic acid in mussel hemocytes exposed to cadmium. Mar. Environ. Res. 85: 13-20, 2013.

Charissou AM, Cossu-Leguille C, Vasseur P. Relationship between two oxidative stress biomarkers, malondialdehyde and 8-oxo-7, 8-dihydro-2'-deoxyguanosine, in the freshwater bivalve *Unio tumidus*. Sci. Total Environ. 322: 109-122, 2004.

Chatziargyriou V, Dailianis S. The role of selenium-dependent glutathione peroxidase (Se-GPx) against oxidative and genotoxic effects of mercury in haemocytes of mussel

Mytilus galloprovincialis (Lmk.) Toxicol. in Vitro 24: 1363-1372, 2010.

Company R, Serafim A, Bebianno MJ, Cosson R, Shillito B, Fiala-Medioni A. Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar. Environ. Res. 58: 377-381, 2004.

Compeau GC, Bartha R. Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. Appl. Environ. Microbiol. 50: 498-502, 1985.

Cunha I, Mangas-Ramirez E, Guilhermino L. Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*). Comp. Biochem. Physiol. 145C: 648-657, 2007.

Fang Y, Yang H, Liu B. Tissue-specific response of metallothionein and superoxide dismutase in the clam *Mactra veneriformis* under sublethal mercury exposure. Ecotoxicology 21: 1593-1602, 2012.

Fang Y, Yang H, Liu Bao, Zhang L. Transcriptional response of lysozyme, metallothionein, and superoxide dismutase to combined exposure to heavy metals and bacteria in *Mactra veneriformis*. Comp. Biochem. Physiol. 157C: 54-62, 2013.

Faria M, Carrasco L, Diez S, Riva MC, Bayona JM, Barata C. Multi-biomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to polychlorobiphenyls and metals. Comp. Biochem. Physiol. 149C: 281-288, 2009.

Frenzilli G, Bocchetti R, Pagliarecci M, Nigro M, Annarumma F, Scarcelli V, *et al.* Time-course evaluation of ROS-mediated toxicity in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. Mar. Environ. Res. 58: 609-613, 2004.

Fridovich I. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64: 97-112, 1995.

Giarratano E, Duarte CA, Amin OA. Biomarkers and heavy metal bioaccumulation in mussels transplanted to coastal waters of the Beagle Channel. Ecotox. Environ. Safe. 73: 270-279, 2010.

Giguere A, Couillard Y, Campbell PGC, Perceval O, Hare L, Pinel-Alloul B, *et al.* Steady-state distribution of metals among metallothionein and other cytosolic ligands and links to cytotoxicity in bivalves living along a polymetallic gradient. Aquat. Toxicol. 64: 185-200, 2003.

Giuliani ME, Benedetti M, Arukwe A, Regoli F. Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. Aquat. Toxicol. 134-135: 120-127, 2013.

Gonzalez PM, Abele D, Puntarulo S. Exposure to excess dissolved iron in vivo affects oxidative status in the bivalve *Mya arenaria*. Comp. Biochem. Physiol. 152C: 167-174, 2010.

Gregory MA, Marshall DJ, George RC, Anandraj A, McClurg TP. Correlations between metal uptake

in the soft tissue of *Perna perna* and gill filament pathology after exposure to mercury. Mar. Pollut. Bull. 45: 114-125, 2002.

Hoarau P, Garello G, Gnassia-Barelli M, Roméo M, Girard JP. Purification and partial characterization of glutathione S-transferases from the clam *Ruditapes decussatus*. Eur. J. Biochem. 269: 4359-4366, 2002.

Irato P, Santovito G, Cassini A, Piccinni E, Albergoni V. Metal accumulation and binding protein induction in *Mytilus galloprovinvialis*, *Scapharca inaequivalis*, and *Tapes philippinarum* from the Lagoon of Venice. Arch. Environ. Contam. Toxicol. 44: 476-484, 2003.

Ji C, Wu H, Liu X, Zhao J, Yu J, Yin X. The influence of salinity on toxicological effects of arsenic in digestive gland of clam *Ruditapes philippinarum* using metabolomics. Chin. J. Oceanol. Limnol. 31: 345-352, 2013.

Ji J, Choi HJ, Ahn IY. Evaluation of manila clam *Ruditapes philippinarum* as a sentinel species for metal pollution monitoring in estuarine tidal flats of Korea: effects of size, sex, and spawning on baseline accumulation. Mar. Pollut. Bull. 52: 447-468, 2006.

Jiang GB, Shi JB, Feng XB. Mercury pollution in China. Environ. Sci. Technol. 40: 3672-3678, 2006.

Jo PG, Choi YK, Choi CY. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. Comp. Biochem. Physiol. 147C: 460-469, 2008.

Kim KY, Lee SY, Cho YS, Bang IC, Kim KH, Kim DS, *et al.* Molecular haracterization and mRNA expression during metal exposure and thermal stress of copper/zinc- and manganese-superoxide dismutases in disk abalone, *Haliotis discus discus*. Fish Shellfish Immunol. 23: 1043-1059, 2007.

Kim WS, Hur HT, Hur SH, Lee TW. Effects of salinity on endogenous rhythm of the manila clam *Ruditapes philippinarum* (Bivalvia: Veneridae). Mar. Biol. 138: 157-162, 2001.

Korashy HM, El-Kadi AO. The role of redox-sensitive transcription factors NF-kappaB and AP-1 in the modulation of the Cyp1a1 gene by mercury, lead, and copper. Free Radic. Biol. Med. 44: 795-806, 2008.

Laing I, Child AR. Comparative tolerance of small juvenile palourdes (*Tapes decussates* L.) and manila clams (*Tapes philippinarum* Adams & Reeve) to low temperature. J. Exp. Mar. Biol. Ecol. 195: 267-285, 1996.

Larose C, Canuel R, Lucotte MD, Giulio RT. Toxicological effects of methylmercury on walleye (*Sander vitreus*) and perch (*Perca flavescens*) from lakes of the boreal forest. Comp. Biochem. Physiol. 147C: 139-149, 2008.

Lee KW, Raisuddin S, Rhee JS, Hwang DS, Yu IT, Lee YM, *et al.* Expression of glutathione S-transferase (GST) genes in the marine copepod *Tigriopus japonicus* exposed to trace metals. Aquat. Toxicol. 89: 158-166, 2008.

Li C, Li H, Su X, Li T. Identification and characterization of a clam ferritin from

Sinonovacula constricta. Fish Shellfish Immunol. 30: 1147-1151, 2011.

Liang L, He B, Jiang G, Chen D, Yao Z. Evaluation of mollusks as biomonitors to investigate heavy metal contaminations along the Chinese Bohai sea. Sci. Total Environ. 324: 105-113, 2004.

Liu X, Zhang L, You L, Yu J, Cong M, Wang Q, *et al.* Assessment of Clam *Ruditapes philippinarum* as heavy metal bioindicators using NMR-based metabolomics. Clean - Soil, Air, Water 39: 759-766, 2011a.

Liu X, Zhang L, You L, Yu J, Zhao J, Li L, *et al.* Differential toxicological effects induced by mercury in gills from three pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. Ecotoxicology 20: 177-186, 2011b.

Liu X, Zhang L, You L, Cong M, Zhao J, Wu H, *et al.* Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. Environ. Toxicol. Pharmacol. 31: 323-332, 2011c.

Liu X, Zhao J, Wu H, Wang Q. Metabolomic analysis revealed the differential responses in two pedigrees of clam *Ruditapes philippinarum* towards *Vibrio harveyi* challenge. Fish Shellfish Immunol. 35: 1969-1975, 2013a.

Liu X, Ji C, Zhao J, Wu H. Differential metabolic responses of clam *Ruditapes philippinarum* to *Vibrio anguillarum* and *Vibrio splendidus* challenges. Fish Shellfish Immunol. 35: 2001-2007, 2013b.

Lu Y, Zhang P, Li C, Su X, Jin C, Li Y, *et al.* Characterisation of immune-related gene expression in clam (*Venerupis philippinarum*) under exposure to di(2-ethylhexyl) phthalate. Fish Shellfish Immunol. 34: 142-146, 2013.

Lund BO, Miller DM, Woods JS. Mercury-induced H_2O_2 production and lipid peroxidation in vitro in rat kidney mitochondria. Biochem. Pharmacol. 42: S181-S187, 1991.

Medeiros ID, Siebert MN, Toledo-Silva G, Rodrigues TB, Marques MRF. Bainy ACD induced gene expression in oyster *Crassostrea gigas* exposed to sewage. Environ. Toxicol. Pharmacol. 26: 362-365, 2008.

Moraga D, Mdelgi-Lasram E, Romdhane MS, Abed A, Boutet I, Tanguy A, *et al.* Genetic responses to metal contamination in two clams: *Ruditapes decussatus* and *Ruditapes philippinarum*. Mar. Environ. Res. 54: 521-525, 2002.

Myers CR. The effects of chromium(VI) on the thioredoxin system: implications for redox regulation. Free Radic. Biol. Med. 52: 2091-2107, 2012.

Nasci C, Da-Ros L, Nesto N, Sperni L, Passarini F, Pavoni B. Biochemical and histochemical responses to environmental contaminants in clam, *Tapes philippinarum*, transplanted to different polluted areas of Venice Lagoon. Italy, Mar. Environ. Res. 50: 425-430, 2000.

Nugroho A P, Frank H. Effects of copper on lipid peroxidation, glutathione, metallothionein, and antioxidative enzymes in the freshwater mussel *Anodonta anatina*. Theor. Exp. Chem. 94: 918-929, 2012.

Prakash TJ, Rao KS. Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. Mol. Cell. Biochem. 146: 107-113, 1995.

Quig D. Cysteine metabolism and metal toxicity. Altern. Med. Rev. 3: 262-270, 1998.

Regoli F, Benedetti M, Giuliani ME. Antioxidant defenses and acquisition of tolerance to chemical stress. Tolerance to environmental contaminants. pp:153-173, 2011.

Rhee JS, Lee YM, Hwang DS, Won EJ, Raisuddin S, Shin KH, *et al.* Molecular cloning, expression, biochemical characteristics, and biomarker potential of theta class glutathione S-transferase (GST-T) from the polychaete *Neanthes succinea*. Aquat. Toxicol. 83: 104-115, 2007.

Roméo M, Gnassia-Barelli M. Effect of heavy metals on lipid peroxidation in the mediterranean clam *Ruditapes decussatus*. Comp. Biochem. Physiol. 118C: 33-37, 1997.

Shailaja MS, D'Silva C. Evaluation of impact of PAH on a tropical fish, *Oreochromis mossambicus* using multiple biomarkers. Aquat. Toxicol. 53: 835-841, 2003.

Sobrino-Plata J, Ortega-Villasante C, Flores-Cáceres ML, Escobar C, Del Campo FF, Hernández LE. Differential alterations of antioxidant defenses as bioindicators of mercury and cadmium toxicity in alfalfa. Chemosphere 77: 946-954, 2009.

Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. 62: 787-821, 1993.

Tavazzi B, Pierro DD, Amorini AM, Fazzina G, Tuttobene M, Giardina B, *et al.* Energy metabolism and lipid peroxidation of human erythrocytes as a function of increased oxidative stress. Eur. J. Biochem. 267: 684-689, 2000.

Umasuthan N, Revathy KS, Lee Y, Whang I, Choi CY, Lee J. A novel molluscan sigma-like glutathione S-transferase from Manila clam, *Ruditapes philippinarum*: Cloning, characterization and transcriptional profiling. Comp. Biochem. Physiol. 155C: 539-550, 2012.

Verlecar XN, Jena KB, Chainy GBN. Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.), on mercury exposures. Chemosphere 71: 1977-1985, 2008.

Wang Q, Ning X, Chen L, Pei D, Zhao J, Zhang L, *et al.* Responses of thioredoxin 1 and thioredoxin-related protein 14 mRNAs to cadmium and copper stresses in *Venerupis philippinarum*. Comp. Biochem. Physiol. 154C: 154-160, 2011.

Wang Q, Yuan Z, Wu H, Liu F, Zhao J. Molecular characterization of a manganese superoxide dismutase and copper/zinc superoxide dismutase from the mussel *Mytilus galloprovincialis*. Fish Shellfish Immunol. 34: 1345-1351, 2013.

Wu H, Liu X, Zhao J, Yu J. NMR-Based metabolomic investigations on the differential responses in adductor muscles from two pedigrees of Manila

clam *Ruditapes philippinarum* to cadmium and zinc. Mar. Drugs 9: 1566-1579, 2011.

Wu H, Liu X, Zhang X, Ji C, Zhao J, Yu J. Proteomic and metabolomic responses of clam *Ruditapes philippinarum* to arsenic exposure under different salinities. Aquat. Toxicol. 136-137: 91-100, 2013a.

Wu H, Zhang X, Wang Q, Li L, Ji C, Liu X, *et al.* A metabolomics investigation on arsenic-induced toxicological effects in the clam *Ruditapes philippinarum* under different salinities. Ecotoxicol. Environ. Safe. 90: 1-6, 2013b.

Wu H, Ji C, Wang Q, Liu X, Zhao J, Feng J. Manila clam *Venerupis philippinarum* as a biomonitor to metal pollution. Chin. J. Oceanol. Limnol. 31: 65-74, 2013c.

Yamuna A, Bhavan PS, Geraldine P. Glutathione S-transferase and metallothionein levels in the freshwater prawn *Macrobrachium malcolmsonhii* exposed to mercury. J. Environ. Biol. 33: 133-137, 2012.

Zanette J, Nunes FF, Medeiros ID, Siebert MN, Mattos JJ, Luchmann KH, *et al.* Comparison of the antioxidant defense system in *Crassostrea rhizophorae* and *Crassostrea gigas* exposed to domestic sewage discharges. Mar. Environ. Res. 66: 196-198, 2008.

Zhang H, Pan L, Tao Y. Toxicity assessment of environmental pollutant phenanthrene in clam *Venerupis philippinarum* using oxidative stress biomarkers. Environ. Toxicol. Pharmacol. 37: 697-704, 2014.

Zhang L, Liu X, You L, Zhou D, Wang Q, Wu H, *et al.* Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics. Mar. Environ. Res. 72: 33-39, 2011a.

Zhang L, Liu X, Chen L, You L, Pei D, Cong M, *et al.* Transcriptional regulation of selenium-dependent glutathione peroxidase from *Venerupis philippinarum* in response to pathogen and contaminants challenge. Fish Shellfish Immunol. 31: 831-837, 2011b.

Zhang L, Liu X, You L, Zhou D, Yu J, Zhao J, *et al.* Toxicological effects Induced by cadmium in gills of Manila clam *Ruditapes philippinarum* using NMR-based metabolomics. Clean-Soil, Air, Water 39: 989-995, 2011c.

Zhang L, Liu X, You L, Zhou D, Wang Q, Li F, *et al.* Benzo(a)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (^{1}H NMR) based metabolomics. Environ. Toxicol. Pharmacol. 32: 218-225, 2011d.

Zhang L, Liu X, Chen L, Wang Q, Zhao J, You L, *et al.* Molecular cloning and differential expression patterns of sigma and omega glutathione S-transferases from *Venerupis philippinarum* to heavy metals and benzo[a]pyrene exposure. Chin. J. Oceanol. Limnol. 30: 413-423, 2012a.

Zhang L, Qiu L, Wu H, Liu X, You L, Pei D, *et al.* Expression profiles of seven glutathione S-transferase (GST) genes from *Venerupis philippinarum* exposed to heavy metals and benzo[α]pyrene. Comp. Biochem. Physiol. 155C: 517-527, 2012b.

Zhang L, Gan J, Ke C, Liu X, Zhao J, You L, *et al.* Identification and expression profile of a new cytochrome P450 isoform (CYP414A1) in the hepatopancreas of *Venerupis* (*Ruditapes*) *philippinarum* exposed to benzo[α]pyrene, cadmium and copper. Environ. Toxicol. Pharmacol. 33: 85-91, 2012c.

Zhang X. Investigation of pollution of Pb, Cd, Hg, As in seawater and deposit of Bohai Sea area. Heilongjiang Environ. J. 25: 87-90, 2001.