

Glutathione S-transferase (*GST*) gene expression profiles in two marine bivalves exposed to BDE-47 and their potential molecular mechanisms*

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Abstract Glutathione S-transferases (GSTs) are phase II enzymes that facilitate the detoxification of xenobiotics and play important roles in antioxidant defense. We investigated the expression patterns of seven *Venerupis philippinarum* GSTs (*VpGSTs*) and four *Mytilus galloprovincialis* GSTs (*MgGSTs*) following exposure to BDE-47. Differential expressions of the seven *VpGSTs* and four *MgGSTs* transcripts were observed, with differences between the hepatopancreas and gills. Among these GSTs, the sigma classes (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*, *MgGST1*, and *MgGST3*) were highly expressed in response to BDE-47 exposure, demonstrating their potential as molecular biomarkers for environmental biomonitoring studies. We obtained the three-dimensional crystal structures of *VpGSTs* and *MgGSTs* by homologous modeling. A model to elucidate the binding interactions between the ligands and receptors was defined by molecular docking. Hydrophobic and π were the most often observed interactions between BDE-47 and the GSTs.

Keyword: glutathione S-transferase (GST); *Venerupis philippinarum*; *Mytilus galloprovincialis*; 2,2',4,4'-tetrabromodiphenyl ether (BDE-47); molecular docking; biomarker

1 INTRODUCTION

Aquatic organisms are frequently exposed to numerous environmental pollutants (Xue and Warshawsky, 2005; Wan et al., 2009). In recent years, research on detoxification and antioxidant enzyme gene expression has become a powerful tool in assessing the ecotoxicological effects of certain chemicals (Won et al., 2011). Glutathione S-transferases (GSTs) are a group of multifunctional enzymes whose major functions involve the detoxification of both endogenous and xenobiotic compounds such as ROS, intracellular transport, biosynthesis of hormones, and protection against oxidative stress (Enayati et al., 2005). GSTs play a critical role in mitigating oxidative stress in all life forms (Lee et al., 2006) and their activities are widely used as biomarkers for environmental monitoring (Shallaja and D'Silva, 2003;

Hoarau et al., 2006; Cunha et al., 2007).

GSTs are found in many prokaryotic and eukaryotic organisms in three intracellular locations: the cytosol, mitochondria, and microsomes (Hayes et al., 2005). In previous studies, multiple specific *GST* mRNA expressions were investigated in several aquatic organisms following exposure to environmental pollutants (Boutet et al., 2004; Lee et al., 2008; Kim et al., 2010; Nair and Choi, 2011; Won et al., 2011). These studies provided opportunities to explore the correlation of specific *GST* responses with the detoxification of various toxicants; these were useful to evaluate the potential of different *GST* isoforms as molecular

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biomarkers to monitor marine environmental pollution.

Venerupis philippinarum and *Mytilus galloprovincialis* are economically important fishery species in China and are widely distributed in high densities around the heavily polluted Bohai Sea (Liang et al., 2004). They are listed as sentinel organisms in the Mussel Watch Programs launched in China (Conrad et al., 2014). They can accumulate high amounts of contaminants and are used as bioindicators in marine environmental toxicology.

In our previous study, we reported the identification, characterization, and distribution of seven *V. philippinarum* (*VpGSTs*: *VpGSTS1*, *VpGSTS2*, *VpGST3*, *VpGSTO*, *VpGSTMi*, *VpGSTM*, and *VpGSTR*) and four *M. galloprovincialis* (*MgGSTs*: *MgGSTa*, *MgGST1*, *MgGST2*, and *MgGST3*) *GSTs* from our ESTs database (Zhang et al., 2012). These investigations demonstrated that this species exhibits distinct biochemical and genetic responses when exposed to heavy metals and benzo[a]pyrene (Liu et al., 2011; Zhang et al., 2011a, b), possessing the requisite features of a promising indicator species. *GST* isoenzymes are considered useful biomarkers in mollusk species (Boutet et al., 2004; Hoarau et al., 2006; Wan et al., 2008, 2009); investigations on *GST* isoform expression profiles in response to specific environmental pollutants would be helpful in identifying potential biomarkers for future environmental biomonitoring.

In this study, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) was selected as a typical environmental contaminant in the Bohai Sea (Tian et al., 2010). BDE-47 is one of the dominant PBDE congeners with relatively high concentrations in the aquatic environment and human tissues, although it is no longer produced and constitutes only a minor proportion of historical global PBDE production and usage (Darnerud, 2003; Mazdai et al., 2003; Hites, 2004; Schechter et al., 2007). Based on the analysis of PBDE residue levels in human samples from China, BDE-47 and -153 are the dominant PBDE congeners, accounting for 60% of the total (Bi et al., 2006). Here, we investigate expression patterns of seven *VpGSTs* and four *MgGSTs* following exposure to BDE-47.

We obtained the three-dimensional crystal structures of the *VpGSTs* and *MgGSTs* by homologous modeling. A model to elucidate the binding interactions between ligands and receptor interactions was defined by molecular docking—which has become an integral part of many modern structure-based computational chemical simulations (Martinez

et al., 2008). Docking methodologies use the knowledge of the three-dimensional structure of a receptor in an attempt to optimize the bound ligand or a series of molecules into the active site. Molecular docking can provide more information on the molecular toxicological mechanism of BDE-47 (Sipl, 2002).

2 MATERIAL AND METHOD

2.1 Animal culture and treatments

The clams, *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees), and adult mussels, *M. galloprovincialis* (shell length: ~4 cm), were purchased from a local market and acclimatized in aerated seawater at 25°C for 10 days prior to BDE-47 exposure. During the acclimatization period, the clams were fed *Chlorella vulgaris* Beij, and the water was renewed daily. After acclimatization, the clams and mussels were randomly divided into flat-bottomed rectangular tanks, each containing 20 individuals in 10 L seawater. During the experiment, the clams and mussels were fed *Isochrysis galbana* and *Platymonas helgolandica*, and the seawater was renewed daily.

For the exposure experiment, all 20 clams or mussels per tank were individually treated with the toxicant BDE-47 at 10 µg/L dissolved in DMSO, a concentration previously reported from some heavily polluted sites in the Bohai Sea and the coastal waters of southeast China (Jin et al., 2008; Wan et al., 2008; Pan et al., 2010). The clams and mussels cultured in normal filtered seawater (FSW) and FSW containing 0.002% DMSO (v/v) were used as the blank and control groups, respectively. During exposure, the clams were fed *Isochrysis galbana* and *Platymonas helgolandica* in FSW for 2 h and then transferred into renewed toxicant-laden seawater daily. The hepatopancreas and gills of six individuals from each treatment were sampled separately after 96-h exposure. The samples were immediately flash-frozen in liquid nitrogen and stored at -80°C for subsequent total RNA extraction.

2.2 Total RNA extraction

Total RNA was extracted from different tissues by TRIZOL[®] Reagent following the manufacturer's directions (Invitrogen life technologies, USA). First-strand cDNA synthesis was carried out using M-MLV RT (Promega, USA) according to the manufacturer's instructions, oligo(dT)-adaptor(5'-CTCGAGATCGA-TGCGGCCGCT17-3') as the primer, and the Dnase

Table 1 Primer sequences used in the present study

Gene	GenBank accession No.	Oligo name	Sequence (5'→3')	Remarks
<i>VpGSTS1</i>	AEW46325	F	CAGAAGAATTTGGCAGAAGTAG	Real-time PCR
		R	AAGACAGCAAGATCAGCGAG	
<i>VpGSTS2</i>	AEW46326	F	AAGGCTAAACTTACAGAGGAG	Real-time PCR
		R	GTGTTTCTTGAGTTCAGGGT	
<i>VpGST3</i>	AEW46327	F	AGATGTATGACAGCGTTGGTG	Real-time PCR
		R	CTGGTTCGGTTCCATAGTG	
<i>VpGSTO</i>	AEW46328	F	CAAGAAAGGAGGGAAAGGAGAT	Real-time PCR
		R	CAGCGAAGAAATGAGCATAACG	
<i>VpGSTMi</i>	AEW46329	F	ACCAGCGGACTTCAGTATCC	Real-time PCR
		R	TTCCTGCCTGTCACCTGTT	
<i>VpGSTM</i>	AEW46330	F	GACTTCCCAATGTACGAGCTT	Real-time PCR
		R	ACACTTTCCTGAGCGAGATAC	
<i>VpGSTR</i>	AEW46331	F	CCACCACATTGGAAGAAGGG	Real-time PCR
		R	ATGGCACCGAGTCAGTTCAG	
<i>MgGSTa</i>	JX485635	F	ATCAGGAGGCTGCCAAAGTA	Real-time PCR
		R	CTACAGCCAACAGGCACTCA	
<i>MgGST1</i>	JX485636	F	GGAGCTGGCTCGTATCATGT	Real-time PCR
		R	TGCAATGGCCATAGACTGAG	
<i>MgGST2</i>	JX485637	F	TGAAGCAAAGAAGGCAGAGG	Real-time PCR
		R	CAAGCCTTACATCCGGGTA	
<i>MgGST3</i>	JX485738	F	AAGGAAAAGGAAGGGGTGAA	Real-time PCR
		R	GAAATGGCACCCTCTGGTT	

I-treated (Promega) total RNA as a template (Chen et al., 2011).

2.3 *VpGST* and *MgGST* mRNA expressions in different tissues following BDE-47 exposure

VpGST and *MgGST* mRNA expressions and the response to BDE-47 were measured by quantitative real-time RT-PCR in an Applied Biosystems® 7500 fast Real-time PCR System (Applied Biosystems, USA). Details of the primers used for RT-PCR are given in supplemental Table 1. In a 96-well plate, each sample was run in triplicate along with the internal control. The PCR amplification was carried out in a total volume of 50 µL, containing 25 µL of 2×SYBR® Green PCR Master Mix (Applied Biosystems), 20 µL of the diluted cDNA, 0.5 µL of each primer (10 µmol/L), and 4 µL of DEPC-treated water. The thermal profile for RT-PCR was 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 31 s. At the end of each PCR reaction, a dissociation curve analysis of amplification products was performed to confirm that

only one PCR product was amplified and detected. Moreover, each real-time RT-PCR product was purified and sequenced to verify the PCR specificity. After amplification, data acquisition and analysis were performed using Sequence Detection Software v2.3 (Applied Biosystems). The $2^{-\Delta\Delta C_t}$ method was chosen as the calculation method. All datasets are given in terms of relative mRNA expression as means \pm standard deviation. One-way analysis of variance (ANOVA) was performed on all data in SPSS 13.0, and $P < 0.05$ was considered statistically significant.

2.4 Homologous modeling and molecular simulation for binding interaction

Because of its persistence and ability to bioaccumulate, PBDE metabolism in humans and other mammals typically occurs via oxidative pathways, producing hydroxylated PBDEs (HO-PBDEs) and brominated phenols (Stapleton et al., 2009; Li et al., 2010). HO-PBDEs are formed from the corresponding PBDEs in the presence of cytochrome P450 enzymes (CYPs) in both humans

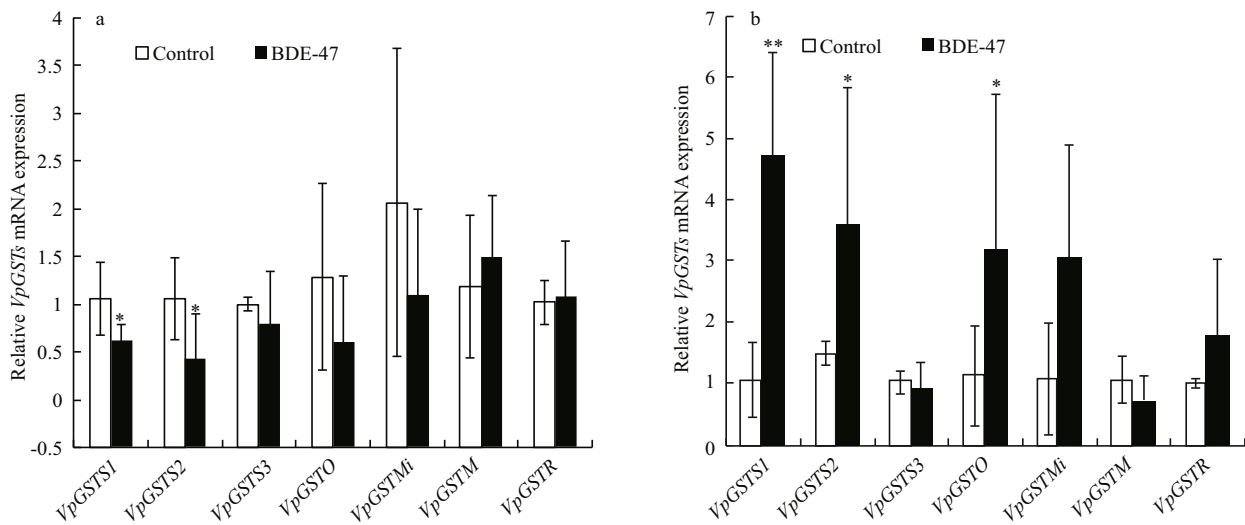


Fig.1 *VpGST* isoform (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*, *VpGSTMi*, *VpGSTM*, and *VpGSTR*) mRNA expression profiles in (a) the hepatopancreas and (b) the gills following treatment with 10 µg/L BDE-47 for 96 h

VpGSTs mRNA expression level values were calculated relative to actin expression and are shown as means±standard deviations ($n=4$). Asterisks indicate significant differences (* $P<0.5$, ** $P<0.05$) compared with the control.

and animals as well as chemically in the atmosphere. GSTs are responsible for phase II conjugation of many xenobiotics with glutathione and are capable of catalyzing reductive dehalogenation reactions, as evidenced by the reductive dechlorination of DDT by *Drosophila* GST (Tang and Tu, 1994).

We carried out homologous sequence searches in BLAST, and sequence alignment was completed in Clustal-W (Ramos et al., 2001). A homologous 3D model of *VpGSTs* and *MgGSTs* was built on the SWISS-MODEL net server. The binding interactions were studied by CDOCKER, which has been incorporated into Discovery Studio 2.5 (Accelrys Software Inc.) through the Dock Ligands protocol. CDOCKER is an implementation of the docking tool based on the CHARMM force field; its viability has been proven (Wu et al., 2003). In CDOCKER, random ligand conformations are generated through molecular dynamics and a variable number of rigid-body rotations/translations are applied to each conformation to generate the initial ligand poses. Grid-based simulated annealing in the receptor active site further refines the conformations, making the results accurate. From the docking analysis, we gained insights into the interactions between the ligands and the receptor.

3 RESULT

3.1 Effect of BDE-47 on different *VpGST* isoform expressions

GST messenger RNA expressions in *V.*

philippinarum hepatopancreas and gills exhibited differential expression patterns following exposure to 10 µg/L BDE-47 for 96 h (Fig.1). Among the different sigma class *VpGSTs* in the hepatopancreas (Fig.1a), the expression levels of *VpGSTS1* and *VpGSTS2* were significantly down-regulated (less than two-fold) after being treated with 10 µg/L BDE-47 for 96 h ($P<0.5$). However, *VpGSTS1* and *VpGSTS2* expression levels in the gills increased 4.47-fold and 2.38-fold ($P<0.05$), respectively, compared with that of the control group after 96 h of 10 µg/L BDE-47 exposure (Fig.1b). *VpGSTO* mRNA expression levels also increased significantly (2.81-fold) under the same treatment (Fig.1b).

3.2 Effect of BDE-47 on different *MgGST* isoform expressions

MgGST mRNA expressions in BDE-47-exposed *M. galloprovincialis* exhibited significantly different expression profiles (Fig.2). *MgGSTS1* expression in the hepatopancreas increased 4.49-fold ($P<0.05$) (Fig.2a) and *MgGSTS3* in the gills dropped 0.69-fold ($P<0.5$) compared with those of the control group (Fig.2b) following exposure to 10 µg/L of BDE-47 for 96 h. However, no significant transcription changes were observed in the other *MgGST* isoforms (Fig.2).

The expression profiles of seven *VpGST* and four *MgGST* genes were investigated to identify promising *GST* biomarkers. Among the three sigma class *VpGSTs*, *VpGSTS2* was significantly down-regulated

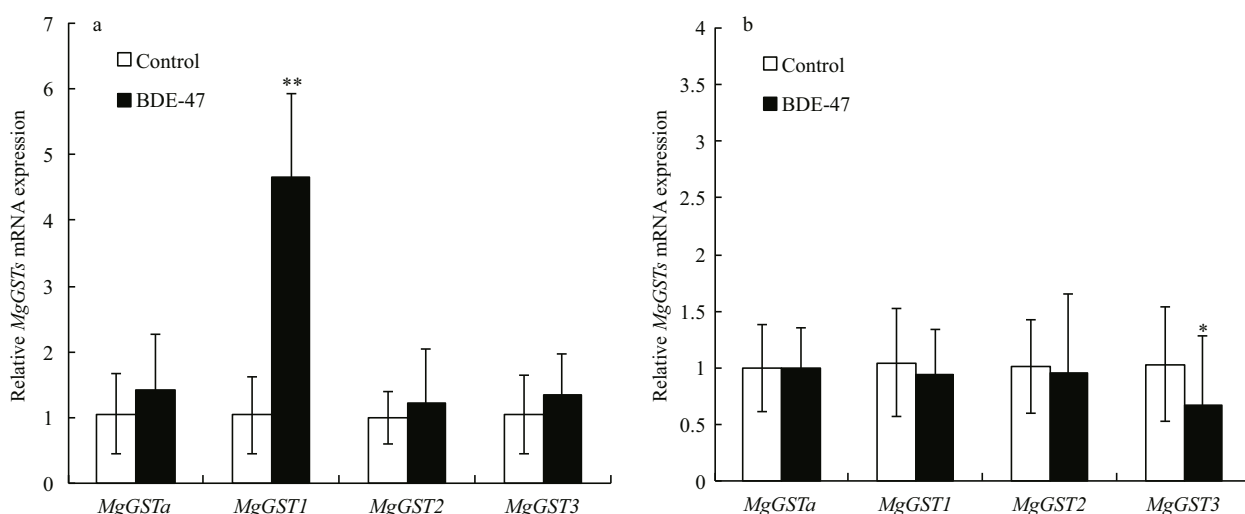


Fig.2 *MgGST* isoform (*MgGSTa*, *MgGSTS1*, *MgGSTS2*, and *MgGSTS3*) mRNA expression profiles in (a) the hepatopancreas and (b) the gills following treatment with 10 µg/L BDE-47 for 96 h

The *MgGST* mRNA expression level values were calculated relative to actin expression and are shown as means±standard deviations (n=4). Asterisks indicate significant differences (* *P*<0.5, ** *P*<0.05) compared with the control.

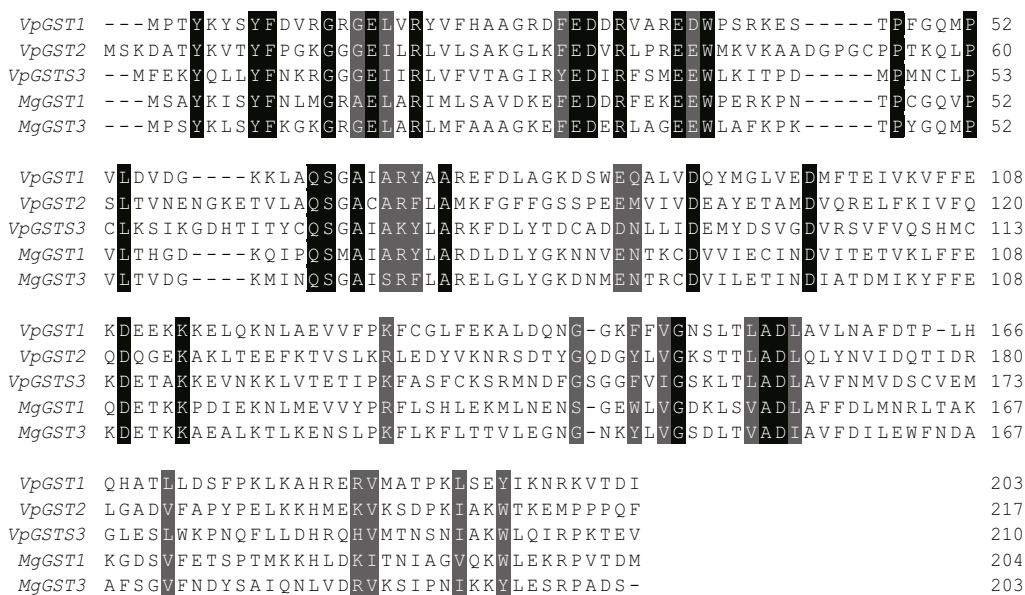


Fig.3 *VpGST* (*VpGSTS1*, *VpGSTS2*, and *VpGSTS3*) and *MgGST* (*MgGST1* and *MgGST3*) sequence alignments

in the hepatopancreas, while *MgGSTS1* was significantly up-regulated after BDE-47 exposure. *VpGSTS1* and *VpGSTS2* expression increased dramatically in the gills, while *MgGSTS3* displayed notable decreases (*P*<0.5).

3.3 Molecular docking

Homologous sequence searches were again carried out in BLAST for three *VpGSTs* (*VpGSTS1*, *VpGSTS2*, and *VpGSTS3*) and two *MgGSTs* (*MgGST1* and *MgGST3*) and the sequence alignment was completed in Clustal-W (Fig.3). The sequence consistency of

VpGSTS1 and *MgGST1* was 41.2% and the sequence similarity was as high as 70.6%.

In this study, *VpGSTS1* and *MgGST1* were selected to investigate the binding interaction with BDE-47. A homologous 3D model of *VpGSTs* and *MgGSTs* was built on the SWISS-MODEL net server. In general, GST isoenzymes can accommodate a broad range of electrophilic substrates. However, some isoenzymes display a relatively high stereoselectivity (deGroot et al., 1996). Each GST isoenzyme subunit contains an active site consisting of two binding sites: the N-terminal domain adopts a beta fold and contains a

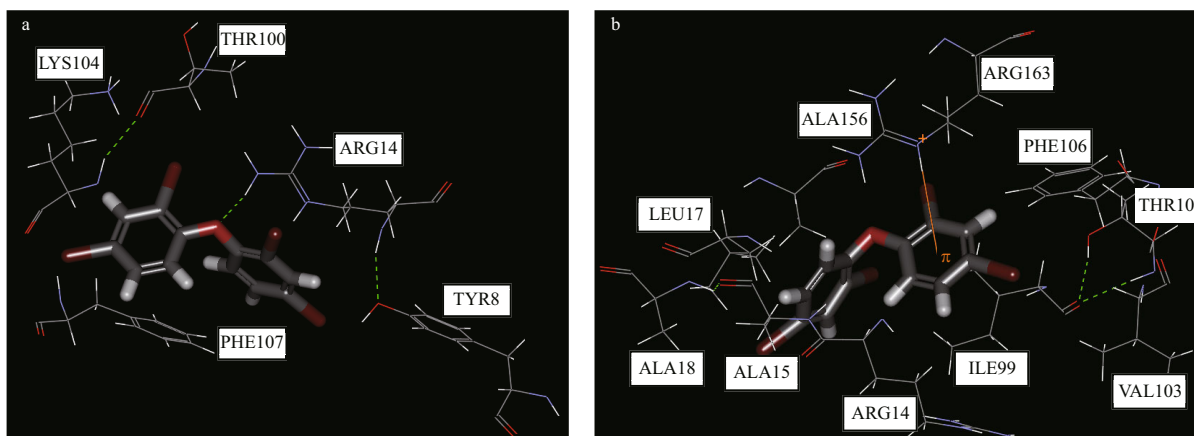


Fig.4 Docking views of (a) *VpGSTS1* vs. BDE-47 and (b) *MgGSTI* vs. BDE-47 in the GST binding sites

Green dotted line indicates H-bonds between BDE-47 and amino acid residues. Orange lines indicate the π interactions between BDE-47 and receptors. Gray: carbon; red: oxygen; blue: nitrogen.

GSH binding site (G-site). The C-terminal domain is mostly helical and provides a hydrophobic cavity (H-site) for binding electrophilic substrates (Danielson and Mannervik, 1985; deGroot et al., 1996). Although the G-site is highly homologous in all of the GSTs, there is a great deal of variability in the H-site, which confers the differing substrate selectivity and catalytic properties for each gene class. The docking view of BDE-47 in the *VpGSTS1* and *MgGSTI* binding sites are shown in Fig.4.

Hydrogen-bonding, hydrophobic- and π -interactions were characteristic of BDE-47 and the GST interactions. At the deep end of the pocket, Arg14 and Phe107 served as anchoring points for the ligands. An H-bond was observed between the oxygen of BDE-47 and the hydrogen of Arg14 (*VpGSTS1* numbering) (Fig.4a). There were also π - π and π -cation interactions between the phenyl of BDE-47 and Phe107 and Arg14 (*VpGSTS1* numbering) (Fig.4a). π -cation interactions were also observed in the BDE-47 and *MgGSTI* binding (Fig.4b). However, there were no bindings between BDE-47 and either *VpGSTMi*, *VpGSTM*, or *VpGSTR*. From the homology modeling and docking data, the difference in stereoselectivity among GSTs for BDE-47 may be the result of different responses to changes in GST mRNA.

4 DISCUSSION

The molecular biological approach in studying potential biomarkers has been widely used as a reliable means to conduct environmental monitoring studies (Hoarau et al., 2006; Rhee et al., 2007). Since GSTs play important roles in both detoxification

pathways and the oxidative stress response, they have been widely investigated in ecotoxicology and marine pollution assessment (Boutet et al., 2004; Hoarau et al., 2006; Kim et al., 2010). Therefore, changes in *VpGST* and *MgGST* expressions observed in *V. philippinarum* and *M. galloprovincialis* exposed to BDE-47 indicate that GSTs might play a defensive role against BDE-47 toxicity. However, as a biomonitor, the information on various classes of *V. philippinarum* and *M. galloprovincialis* GSTs and their spatial and temporal expression profiles following BDE-47 exposure is currently unavailable.

As important phase II biotransformation enzymes, GSTs catalyze the conjugation of glutathione (GSH) to electrophilic xenobiotics and oxidized components to increase their hydrophilicity and facilitate the excretion of toxicants (Ketterer et al., 1983). Chemical exposure may influence transcription regulatory factors that bind with the promoter region of some GST genes and mediate the transcription of specific GST isoenzymes (Kim et al., 2010). Moreover, accumulation of GSH conjugation with chemicals—an inhibitor of GST expression—might result in significant GST down-regulation (Srivastava et al., 1999).

Seven *VpGST* gene expression profiles were studied to identify potential GST biomarkers. In the hepatopancreas, among the *VpGSTs*, *VpGSTS1* and *VpGSTS2* were significantly down-regulated, while *VpGSTM* was significantly up-regulated following exposure to 10 $\mu\text{g/L}$ BDE-47 for 96 h. However, *VpGSTS1*, *VpGSTS2*, *VpGSTO*, and *VpGSTMi* were significantly up-regulated following BDE-47 exposure. Sigma class GSTs may participate in

cellular xenobiotic defenses and antioxidant systems in various organisms (Singh et al., 2001; Lee et al., 2008; Won et al., 2011). For example, *Tigriopus japonicus* GST class sigma (*GST-S*) is more consistent than other types of *GST* when exposed to hydrogen peroxide and heavy metals (Lee et al., 2008). A sigma *GST* from disk abalone (*HdGSTS1*) also exhibits considerable inducibility by organic contaminants (Wan et al., 2008). Recent studies have shown that sigma *GSTs* from *Chironomus riparius* and *Perinereis nuntia* display great potential as biomarkers for cadmium and silver nanoparticle exposure (Won et al., 2011). The results in the present study were consistent with previous reports, clearly demonstrating that some sigma class *VpGSTs* are more sensitive to BDE-47 exposure than other *GSTs*. They also highlight their potential as molecular biomarkers for environmental biomonitoring studies.

GST omega isoenzymes take part in the cellular response to oxidative stress (Board et al., 2000). As reported, the omega *GST* expressions in *Tigriopus japonicus*, *Takifugu obscurus*, *Perinereis nuntia*, and *Haliotis discus discus* are significantly induced by Cd or Cu exposure (Lee et al., 2008; Wan et al., 2009; Kim et al., 2010; Won et al., 2011). In this work, *VpGSTO* in the gills increased dramatically in response to BDE-47. In the case of *VpGSTMi*, a similar pattern of transcriptional repression was observed following BDE-47 exposure. Microsomal *GST* is ubiquitously expressed and predominantly located in the endoplasmic reticulum and outer mitochondrial membrane; it is vital for the protection of organisms against agents known to induce lipid peroxidation (Johansson et al., 2010). Furthermore, many organisms overexpress *GSTs* on exposure to chemicals (Hayes et al., 2005).

MgGSTS1 expression in the hepatopancreas increased significantly, while *MgGSTS3* in the gills dropped after 96-h exposure to 10 µg/L of BDE-47. *MgGST* mRNA expressions in BDE-47-exposed *M. galloprovincialis* exhibited significantly different expression profiles. Of the four *MgGSTs*, the sigma class (*MgGSTI*) was more sensitive to BDE-47 exposure than the other *GSTs*.

Taken together, our results show that BDE-47 exposure caused differential expressions in the seven *VpGSTs* and four *MgGSTI* transcripts. The selective up- or down-regulation of *VpGST* gene expression highlights the fact that these isoenzymes probably play divergent physiological roles during the detoxification of various pollutants in *V. philippinarum*

and *M. galloprovincialis*. Among these *GSTs*, the sigma classes (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*, *MgGSTI*, and *MgGST3*) were highly expressed in response to BDE-47 exposure, demonstrating their enormous potential as molecular biomarkers for environmental biomonitoring studies.

5 CONCLUSION

In summary, we investigated the expression patterns of seven *VpGSTs* and four *MgGSTs* following exposure to BDE-47. The three-dimensional crystal structures of the *VpGSTs* and *MgGSTs* were obtained by homologous modeling. From the molecular docking study, hydrogen-bonding, hydrophobic- and π -interactions were characteristic of the interactions between BDE-47 and the *GSTs*. *GST* mRNA expressions caused by BDE-47 exposure differed between the hepatopancreas and gills. *VpGST* transcript expressions appeared to be selectively up- or down-regulated, which enhanced our understanding of the antioxidant roles of *VpGSTs* and their biomarker potential. Among these *GSTs*, the sigma classes (*VpGSTS1*, *VpGSTS2*, *VpGSTS3*, *MgGSTI*, and *MgGST3*) were highly expressed in response to BDE-47 exposure, indicating their good biomarker potential for BDE-47 pollution in the marine environment.

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