The role of GST omega in metabolism and detoxification of arsenic in clam *Ruditapes philippinarum*

Lizhu Chen[a,c], Huifeng Wu[a,b,*], Jianmin Zhao[a], Wei Zhang[d], Li Zhang[d], Shan Sun[c], Dinglong Yang[a], Bo Cheng[e], Qing Wang[a,*]

[a] *Key Laboratory of Coastal Zone Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, PR China*
[b] *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, PR China*
[c] *Shandong Marine Resource and Environment Research Institute, Yantai 264006, PR China*
[d] *Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China*
[e] *Aquatic Products Quality and Standards Research Center, Chinese Academy of Fishery Sciences, Beijing 100141, PR China*

ARTICLE INFO

ABSTRACT

The major hazard of arsenic in living organisms is increasingly being recognized. Marine mollusks are apt to accumulate high levels of arsenic, but knowledge related to arsenic detoxification in marine mollusks is still less than sufficient. In this study, arsenic bioaccumulation as well as the role of glutathione S-transferase omega (GSTΩ) in the process of detoxification were investigated in the *Ruditapes philippinarum* clam after waterborne exposure to As(III) or As(V) for 30 days. The results showed that the gills accumulated significantly higher arsenic levels than the digestive glands. Arsenobetaine (AsB) and dimethylarsenate (DMA) accounted for most of the arsenic found, and monomethylarsonate (MMA) can be quickly metabolized. A subcellular distribution analysis showed that most arsenic was in biologically detoxified metal fractions (including metal-rich granules and metallothionein-like proteins), indicating their important roles in protecting cells from arsenic toxicity. The relative mRNA expressions of two genes encoding GSTΩ were up-regulated after arsenic exposure, and the transcriptional responses were more sensitive to As(III) than As(V). The recombinant GSTΩs exhibited high activities at optimal conditions, especially at 37 °C and pH 4–5, with an As(V) concentration of 60 mM. Furthermore, the genes encoding GSTΩ significantly enhance the arsenite tolerance but not the arsenate tolerance of *E. coli* AW3110 (DE3) (Δ*arsRBC*). It can be deduced from these results that GSTΩs play an important role in arsenic detoxification in *R. philippinarum*.

1. Introduction

Arsenic, which naturally exists in the earth's crust, is a highly toxic metalloid. It is known that arsenic can lead to human skin cancer and other diseases (Akter et al., 2005; Jomova et al., 2011). With the increase in ore mining activity, the use of arsenical pesticides and the emission of industrial waste into the sea, arsenic pollution is becoming increasingly serious in coastal areas of China (Luo et al., 2010; Xu et al., 2013; Gao et al., 2014). Arsenic content in offshore marine organisms in China has been recently reported at high concentrations. For example, the total arsenic in the fish *Trypauchen vagina* collected from Zhanjiang (Guangdong, China) was as high as 134 μg/g dry weight (dw) (Zhang and Wang, 2012). It has been widely reported that marine animals such as mollusks, crustaceans and fish typically have high concentrations of

arsenic in their tissues, ranging from less than 1 μg/g dw to more than 100 μg/g dw (Katano et al., 2003; Neff, 1997; Zhang et al., 2013). In marine animal samples, the less toxic organic arsenic constituted most arsenic compounds, and inorganic arsenic generally occurred at levels below 2% for the total arsenic (Li et al., 2003a; Waring and Maher, 2005; Zhang et al., 2013). However, the concentrations of inorganic arsenic in two cultured marine fishes collected from Fujian province (China), *Pagrus major* and *Lateolabrax japonicus*, are up to 2.8 and 4.5 μg/g dw, respectively; the levels are much higher than China's national standard for inorganic arsenic in seafood (0.5 μg/g dw) and may pose a significant threat to human health (Onsanit et al., 2010).

Arsenic has a variety of chemical forms in nature, and its toxicity depends greatly on its chemical species. Marine organisms generally contain high concentrations of arsenic, most of which are present as

nontoxic organic species such as AsB, arsenocholine and arsenosugars (Li et al., 2003a; Fattorini et al., 2004a). Arsenic mainly exists in hypertoxic inorganic arsenic forms of As(III) and As(V) in soil and water (Fattorini et al., 2004a; Francesconi and Edmonds, 1996). General studies show that the toxicity of As(III) is higher than As(V), and methylated pentavalent arsenic compounds such as MMA and DMA are moderately toxic (Rahman and Hassler, 2014). However, recent studies have shown that MMA(III) and DMA(III) exhibit higher toxicity and carcinogenicity than As(III) and As(V) (Akter et al., 2005; Ventura-Lima et al., 2011a).

To date, systematic studies on the molecular mechanism of arsenic metabolism have been done on mammals, bacteria, microalgae and some invertebrates (Qin et al., 2006, 2009; Thomas et al., 2010; Yin et al., 2011). It is generally accepted that the arsenic metabolism process involves successive reductions followed by oxidative methylation reactions (Aposhian et al., 2004; Vahter, 2002), and glutathione-S-transferase omega (GSTΩ) has been shown to be a key enzyme involved in the limiting step of the metabolism process (Sampayo-Reyes and Zakharyan, 2006; Zakharyan and Aposhian, 1999; Zakharyan et al., 2001). For example, human GSTΩ 1-1, identified as a MMA(V) reductase, is able to catalyze the reductions of As(V) to As(III), MMA(V) to MMA(III), and DMA(V) to DMA(III) (Chowdhury et al., 2006; Zakharyan et al., 2001). Moreover, the level of GSTΩ activity has been used as an estimation of the capability of an organism to metabolize inorganic arsenic (Aposhian et al., 2004; Sampayo-Reyes and Zakharyan, 2006; Ventura-Lima et al., 2009, 2011b). At present, GSTΩ homologues have also been widely characterized in marine invertebrates such as polychaetes and mollusks (Ventura-Lima et al., 2011a). However, data on the role of GSTΩ in the detoxification of arsenic in marine organisms are still limited.

Ruditapes philippinarum clams, which are widely distributed in nearshore and estuarine areas, have been widely used in marine ecotoxicology studies because of their high accumulation of metals and other pollutants (Liu et al., 2011). *R. philippinarum* is also one of the bioindicators of the "Mussel Watch Plan" in China, which makes it ideal for studying the bioaccumulation and detoxification mechanism of arsenic. By now, at least five major classes of GSTs (sigma, omega, mu, rho, microsomal) have been characterized in this clam (Zhang et al., 2012b). Distinguished from other subtypes of GST proteins, GSTΩ possesses cysteine residues in its active site, exhibits a number of atypical structural and functional features, and may function as an arsenate reductase during the arsenic detoxification process (Sampayo-Reyes and Zakharyan, 2006; Yin et al., 2001). Additionally, a preliminary transcriptomic study shows that GSTΩs are significantly up-regulated after arsenic exposure, while the transcripts of other GSTs are not significantly modulated (unpublished data). Thus, further work is needed to clarify the possible function of GSTΩs in arsenic detoxification in this clam. The aim of this work was to investigate the arsenic bioaccumulation and metabolism of inorganic arsenic in the clam tissues as well as the possible role of GSTΩ in these processes.

2. Materials and methods

2.1. Clam and sample collection

Healthy clams (shell length: 3.0–4.0 cm) obtained from a local culture farm in Yantai (China) were acclimatized in aerated and filtered seawater (13 °C, 31‰, pH 7.9) for 10 days before the exposure experiment. The clams were fed with *Chlorella vulgaris* throughout the acclimatization and experiment, and the seawater was completely changed daily after a 2-hour feeding.

The stock solutions of As(V) and As(III) were prepared with Na_3AsO_4 (Chem Service, USA) and $NaAsO_2$ (Sinopharm Chemical Reagent Co. Ltd, China) at a concentration of 0.1 mg/mL, respectively. The average concentration of arsenic in the water column of Bohai Sea is about 1 μg/L, like that of the oceans (Gao et al., 2014; Neff, 1997).

However, in some seriously polluted areas such as the estuarine areas of southern Bohai Sea, the average dissolved concentration of As in the water was 18.6 μg/L, with a highest value of 347.7 μg/L (Xu et al., 2013). Thus, exposure concentrations of 10 μg/L and 100 μg/L were used in our study to investigate the toxic effects of arsenic on the bivalves in the above area. The clams were randomly divided into fifteen aquaria, and each group contained 30 individuals. Three tanks without any treatment served as the control group, while the other twelve tanks were exposed to As(V) or As(III) at final concentrations of 10 and 100 μg/L, respectively, each with three replicates. After a 30-day exposure, the gills and digestive glands of the clams were collected. The samples for the total RNA extraction were immersed in a TRIzol reagent (Invitrogen, USA) and stored at −80 °C, while the others were directly kept at −80 °C until analysis. The seawater in the control group and exposure groups was also sampled to assess the natural background value of the arsenic.

2.2. Determination of total arsenic content in clam tissues

The total arsenic content in the clam tissues was measured following previously described and validated procedures (Zhang et al., 2012a). The frozen tissue samples were dried at 50 °C to a constant weight and 1 mL of concentrated HNO_3 added (65%, analytical reagent grade, Fisher Scientific). Subsequently, the samples were heated in a water bath at 80 °C until complete digestion and then were diluted to 10 mL with double deionized water. All experiments were tested with certified oyster tissue material (NIST SRM 1566b, National Institute of Standard and Technology, USA). The total arsenic content was determined by an inductively coupled plasma mass spectrometer (ICP-MS) (PerkinElmer, Elan DRCII, USA) with In (115) (1.0 μg/mL, Agilent, USA) as an internal standard solution. The arsenic standard solution was prepared from a stock solution (GBW08611, Chinese National Standards). The total arsenic recovery rate of the certified material was 95.8%. The total arsenic content in the tissues was shown to be μg/g dw with four parallel samples for each group.

2.3. Arsenic speciation analysis

Arsenic speciation in tissues was detected using HPLC-UV-HG-AFS (AF-610D2, Beifenruili Analytical Instrument Corp., Beijing, China) following the protocol of Zhang et al. (2015). Five mL of 50% methanol solution were added to the samples, which were then homogenized for 15 min. The homogenates were centrifuged twice (10,000 rpm, 10 min) to obtain a supernatant, which was evaporated to remove the organic solvent, after which it was diluted with double deionized water. Finally, the digests were filtered through 0.45 μm filters for an arsenic speciation analysis. The test conditions were as follows: Anion exchange column: Hamilton PRP-X100 (10 microns, 250 mm x 4.1 mm); mobile phase: 20 mmol/L NH_4HCO_3, 10 mmol/L KCl, pH 10.3; current-carrying: 10% (v/v) HCl; reducing agent: 2% of KBH_4 in 0.2% KOH (w/v); antioxidant: 2% (m/v) $K_2S_2O_8$ in 0.5% KOH (w/v); shielding gas: 800 mL/min; carrier gas: 500 mL/min; Lamp current: 100 mA. There were three repetitions in each group.

Quality assurance was done with analyses of standard reference materials — BCR-627 tuna from the Institute for Reference Materials and Measurements (Geel, Belgium). BCR-627 tuna fish tissue (0.1 g) was used for AsB and DMA analysis. The certified values of AsB and DMA in this certified reference material were 3.90 ± 0.22 μg/g and 0.15 ± 0.02 μg/g, respectively. With our method, we obtained an AsB concentration of 4.41 ± 0.03 g/g (113% recovery, *n* = 6) and a DMA concentration of 0.14 ± 0.02 g/g (93% recovery, *n* = 6). Spikes were also used to confirm the recovery of other As species detected during the speciation analysis. In our study, As(III) recoveries were 78–106%, As(V) recoveries were 82–101%, and MMA recoveries were 87–103%.

2.4. Subcellular arsenic distribution

Five subcellular components, including organelles, heat sensitive proteins (HSP), metallothionein-like proteins (MTLPs), metal-rich granules (MRG) and cellular debris, were fractionated by differential centrifugation to determine the subcellular arsenic distribution (Wallace and Luoma, 2003; Zhang et al., 2015). After the samples were weighed, the clam tissues were homogenized in a 20 mM Tris − HCl buffer (pH 7.6) infused with 2-mercaptoethanol (5 mM) and phenylmethanesulfonyl fluoride (0.1 mM) on ice. The five fractions were then obtained as described by Zhang et al. (2015). Afterward, the five fractions were digested, and the total arsenic concentration in each fraction was determined by ICP-MS. The SRM 1566b oyster tissue was analyzed to evaluate the accuracy of the method. The sum of the arsenic content in the five fractions was 75–108% of the certified value in the reference material, showing low recovery efficiency. This was mainly caused by a loss of arsenic in different fractions during measurements. The result was shown as a percentage of arsenic in each fraction with a percentage error of lower than 5%. Each group contained four parallel samples.

2.5. Arsenate reductase assay of GSTΩ

GSTs were purified from the gills and digestive glands of the clams using a commercial kit (MagneGST™ Glutathione Particles, Promega, USA). The arsenate reductase activity of GSTΩ was measured using a previous method (Sampayo-Reyes and Zakharyan, 2006; Ventura-Lima et al., 2011b). Briefly, the protein was incubated at 37 °C in 0.1 M Tris − HCl (pH 7.4) containing 0.25 mM NADPH, 0.8 U glutathione reductase, 5 mM GSH, and 40 mM As(V) for 10 min. The reductase activities were monitored spectrophotometrically at 340 nm (U-3900H, Hitachi, Japan) and were shown as the decrement of NADPH. The extinction coefficient of NADPH is 6.22 mM/cm, and each treatment had three or four repeats.

2.6. Quantitation of GSTΩ transcripts

The qRT-PCR reactions were carried out to analyze the expressions of *GSTΩ 1* and *GSTΩ 2* in the gills and digestive glands of the clams (Yang et al., 2010). The total RNA was extracted and used to synthesize the cDNA with an oligo-dT primer (Table 1). After the reverse transcription, the relative mRNA expressions of *GSTΩ*s were assayed in an Applied Biosystem 7500 Real-time PCR System. *β-actin* was used as an internal control to verify the successful transcription and to calibrate the cDNA template (Zhang et al., 2012b). The gene-specific primers for *GSTΩ*s and *β-actin* were listed in Table 1. The reaction conditions were as follows: 50 °C for 2 min, followed by 40 cycles of 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. The quantitative analysis of expressions of *GSTΩ* genes was performed using the $2^{-\triangle\triangle CT}$ method. All data were given as the mean ± S.E. ($n = 4$–6).

2.7. Recombinant expression and purification of GSTΩ 1 and GSTΩ 2

The genes encoding GSTΩ 1 and GSTΩ 2 were amplified by PCR with two pairs of gene-specific primers, with BamH I and Hind III sites at their 5′ ends, respectively, as described in Table 1 (Wang et al., 2012). The sequences were confirmed by Sanger sequencing (Sino-GenoMax, Beijing, China) and were then cloned into pET-28a vectors (Novagen, USA). Afterwards, the recombinant plasmids were overexpressed in *Escherichia coli* BL21 (DE3) (Novagen, USA). The recombinant proteins were purified using HisTrap affinity columns (GE Healthcare, USA) and dialyzed with 20 mM Tris − HCl (pH 8.0) containing 60 mM NaCl and 5 mM dithiothreitol (DTT) (Sampayo-Reyes and Zakharyan, 2006). The peptides were analyzed by SDS-PAGE with separation in a 15% gel followed by Coomassie brilliant blue R250 staining. Moreover, the concentrations of recombinant proteins were measured with the Bradford method. The recombinant proteins were stored at -80 °C until use.

2.8. Polyclonal antibody preparation and western blotting analysis

The polyclonal antibodies of GSTΩs were produced by immunizing the mice (Luye Pharma Inc., Yantai, China) with the recombinant proteins (Yang et al., 2010). The serum of one mouse injected with PBS served as the negative control. Afterwards, the specificity of the antibodies was confirmed by a western blotting analysis. After the proteins were separated by SDS-PAGE, the gels were transferred to PVDF membranes. The membranes were then respectively incubated with polyclonal antibodies (1:1000 dilution) followed by a reaction with alkaline phosphatase (AP)-conjugated secondary goat anti-mouse IgG (1:3000 dilution, Beyotime, China). The western blotting was developed by NBT/BCIP (Beyotime, China) and terminated by washing the PVDF membranes with deionized distilled water.

2.9. Immunohistochemistry

The fresh tissues of the gills and digestive glands were fixed in 4% paraformaldehyde solution, dehydrated in ethanol, embedded in paraffin and sectioned at 5 μm. The endogenous alkaline phosphatase was blocked by incubating the slides in 3% acetic acid for 10 min. For antigen retrieval, the slides were boiled in a 0.01 M citrate buffer at 120°C for 6 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. The slides were incubated with primary polyclonal antibodies of GSTΩ 1 and GSTΩ 2 (1:500) and washed with PBST (0.01 M PBS, 0.05% Tween 20). The slides were then incubated with biotinylated goat anti-mouse IgG (1:200, Solarbio, China) followed by incubation with streptavidin labeled with alkaline phosphatase (1:300, Beyotime, China). The immunostaining was visualized using the AP-Red Kit (ZSGB-BIO Origene, China), and the slides were counterstained with hematoxylin. In negative controls, the primary antibodies were substituted with the serum of a mouse immunized with PBS.

Table 1
Primer sequences used in this study.

Primer	Sequence (5'-3')	Sequence information
oligo-dT	GGCCACGCGTCGACTAGTACT$_{17}$	primer for cDNA synthesis
β-actin-F	CTCCCTTGAGAAGAGCTACGA	qRT-PCR primer for *β*-actin
β-actin-R	GATACCAGCAGATTCCATACCC	qRT-PCR primer for *β*-actin
GSTΩ 1-F	TGTTATCGGCATTAGATCGTGTCG	qRT-PCR primer for GSTΩ 1
GSTΩ 1-R	AAATCTTGAAGCGTCCAGGGTGA	qRT-PCR primer for GSTΩ 1
GSTΩ 2-F	TGGAGGTGAGAAGCCGATGAT	qRT-PCR primer for GSTΩ 2
GSTΩ 2-R	CTCTTCGGAACATCTGGTTACCTT	qRT-PCR primer for GSTΩ 2
GSTΩ 1r-F	GGATCCGCTCTCGGGTCAGGAAGTAAGTAT	Recombinant primer for GSTΩ 1
GSTΩ 1r-R	AAGCTTTCAGTGGTGGTGGTGGTGGTGTTCAAGACCTGCGTCACAATCC	Recombinant primer for GSTΩ 1
GSTΩ 2r-F	GGATCCGGGAAGTTGCGGGTATA	Recombinant primer for GSTΩ 2
GSTΩ 2r-R	AAGCTTTCAGTGGTGGTGGTGGTGGTGGTGCTCCTTTCTTGCCACTACA	Recombinant primer for GSTΩ 2

The analysis of the GSTΩs expressions in the gills and digestive glands was performed by western blotting (Harju et al., 2008). The total protein of the tissues was extracted using a RIPA lysis buffer with PMSF (Beyotime, China). Then, the concentration of soluble proteins was measured and adjusted for conformity with each other. The western blotting analysis was carried out as stated above. β-actin was used as an internal control to calibrate the expression of GSTΩs and was detected using mouse polyclonal antibodies of β-actin (1:500; Beyotime, China).

2.10. Effect of temperature, the substrate concentrations and pH on arsenate reductase activities of recombinant GSTΩs

The influence of the temperature, As(V) concentration (as the substrate) and pH on arsenate reductase activities of recombinant GSTΩs was respectively detected. The test and data analysis were conducted in light of the methods described in section 2.5. The temperatures ranging from 17 °C to 77 °C, As(V) concentrations ranging from 0 mM to 100 mM and pH values ranging from 3.5 to 9.0 were used.

2.11. Complementation of arsenate sensitive E. Coli AW3110 (DE3) (ΔarsRBC) by recombinant plasmids with GSTΩ

The arsenic resistance phenotype of cells expressing *GSTΩ 1* and *GSTΩ 2* genes was determined in *E. coli. E. coli* AW3110 (DE3), which lacks the operon *arsRBC*, was used for arsenate sensitivity assays (Qin et al., 2006). After being made into TSS competent cells, *E. coli* AW3110 (DE3) (Δ*arsRBC*) was transformed with the recombinant plasmids pET-28a-GSTΩ 1, pET-28a-GSTΩ 2 and pET-28a empty vector, respectively. The single colony of *E. coli* AW3110 (DE3) (Δ*arsRBC*) and the transformed strains were inoculated into 5 mL of LB medium (40 μg/mL kanamycin) and incubated at 37 °C overnight. The cultures were diluted to an OD600 of 0.1 with LB + 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and LB + Kanamycin + 0.3 mM IPTG containing different concentrations of As(V) or As(III) (0, 100, 300 μM). The growth of *E. coli* was monitored at an optical density of 600 nm (37 °C) and was recorded every four hours until the cultures reached the stationary phase. The result was expressed as the average of three independent assays with a standard error.

2.12. Bioinformatics and statistical analysis

The nucleotide sequences were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). Statistical analyses were performed using SPSS 16.0 software (SPSS Inc, Chicago, USA). For all data, normality was tested by Kolmogorov-Smirnov test and homogeneity of variance was tested by Levene's test. The differences of the corresponding values were tested by a one-way analysis of variance (ANOVA) followed by a Duncan test. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Bioaccumulation of arsenic in the clam R. philippinarum

The background of arsenic in seawater was 1.07 μg/L, and the mortality of the clams was less than 4% after inorganic arsenic exposure for 30 days. As illustrated in Fig. 1A, the basal total arsenic concentrations in the gills and digestive glands of *R. philippinarum* were 30.30 ± 5.70 μg/g dw and 19.56 ± 3.36 μg/g dw, respectively, and the concentrations of the total arsenic in the digestive glands were significantly lower than those of the gills. A significant increase of arsenic concentration in gills was detected after 100 μg/L As(III) exposure for 30 days ($P < 0.05$), while there was no significant difference in arsenic concentrations between different groups after As(V) exposure. In the digestive glands, significant arsenic accumulation was detected
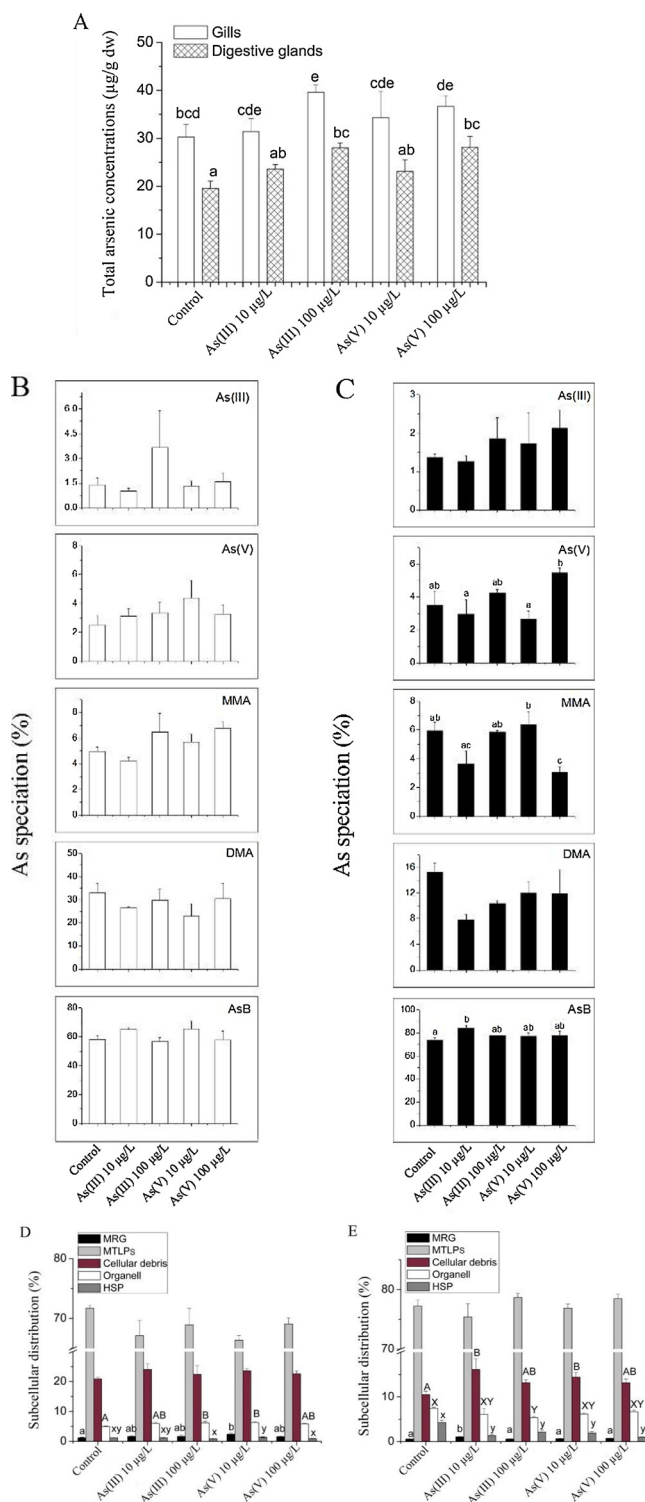
Fig. 1. Arsenic contents and subcellular distribution analysis in tissues. (A) Total As concentrations in tissues after inorganic arsenic exposure ($n = 4$–6). Arsenic percentages of different speciation (%) in gills (B) and digestive glands (C) after arsenic exposure ($n = 3$). The white-shaded and black-shaded columns respectively represent the As percentages of different speciation (%). Arsenic subcellular distribution (%) in gills (D) and digestive glands (E) after arsenic exposure ($n = 4$). Metal-rich granules are shaded in black, metallothionein-like proteins in light gray, cellular debris in burgundy, organelle in white, and heat sensitive proteins in dark gray. Values are shown as means ± SE. Different letters indicate significant differences between the treatments ($P < 0.05$).

along with exposure to 100 μg/L As(III) and 100 μg/L As(V) ($P <$ 0.05). Thus, compared to the gills, arsenic more easily accumulated in the digestive glands of the clam *R. philippinarum*.

3.2. Arsenic species in the clam R. philippinarum

Fig. 1B and C showed the percentages of five arsenic species in the gills and digestive glands of clams, among which AsB (56.73%–84.33%) was the main arsenic species, followed by DMA and MMA. The percentages of inorganic arsenic were relatively small, with As(III) and As(V) accounting for, at most, 3.65% and 5.46% of the total arsenic, respectively. Inorganic arsenic exposure showed no influence on the percentages of all five arsenic species in the gills (Fig. 1B). In digestive glands, the percentages of As(III) and As(V) in the exposure groups did not significantly change compared to the control group, while the proportion of MMA in the 100 μg/L As(V) exposure group decreased significantly ($P <$ 0.05), and the proportion of AsB exhibited a significant increase after 10 μg/L As(III) exposure ($P <$ 0.05) (Fig. 1C). Thus, it is suggested that inorganic arsenic can be effectively converted in organic species in *R. philippinarum* and that MMA can be quickly metabolized or converted into other forms in digestive glands.

3.3. Subcellular distribution in tissues

Arsenic distribution in the subcellular fractions of gills and digestive glands were given in Fig. 1D and 1E. MTLPs (66.36%–78.69%) were the main binding sites of arsenic in cells, followed by cellular debris (10.50%–24.03%). After inorganic arsenic exposure, total arsenic content in cellular debris increased, especially in the digestive glands of 10 μg/L As(III) and As(V) exposure groups ($P <$ 0.05) (Fig. 1E). However, a significant reduction of arsenic content in HSP fraction appeared in the digestive glands ($P <$ 0.05), as well as a slight falling in the organelles (Fig. 1E).

For better prediction of the biological toxicity of arsenic and the detoxification capability of cells, the above five subcellular components can be classified into three subcellular compartments: biologically detoxified metal fraction (BDM, including MRG and MTLPs), metal-sensitive fraction (MSF, including organelles and HSP) and cellular debris. As shown in Fig. 1F and G, although no significant differences were found after inorganic arsenic exposure, the subcellular partitioning of arsenic in BDM was the highest, followed by cellular debris and MSF. Compared to the control groups, arsenic in MSF increased in the gills, especially in 10 μg/L As(V) exposure group ($P <$ 0.05) (Supplementary material, Fig. S1A), but remarkably decreased in digestive glands in all exposure groups ($P <$ 0.05) (Supplementary material, Fig. S1B), indicating the higher detoxification capability of digestive glands compared to gills. Additionally, arsenic in cellular debris fraction increased significantly in digestive glands, particularly in the 10 μg/L As(III) and 10 μg/L As(V) exposure groups ($P <$ 0.05) (Supplementary material, Fig. S1B).

3.4. Arsenate reductase activities and the relative mRNA expressions of GSTΩs in the tissues of R. philippinarum

As shown in Fig. 2A, the arsenate reductase activities of GSTΩ in the digestive glands were remarkably higher than those in gills. However, it was noteworthy that the activities did not change in these tissues after different treatments. For *GSTΩ 1*, significant transcriptional up-regulation appeared in the gills of clams treated with 10 μg/L As(III) (Fig. 2B) and the digestive glands of clams treated with 100 μg/L As(III) (Fig. 2C), respectively. After arsenic exposure, the mRNA expression level of *GSTΩ 2* was remarkably up-regulated in the gills of clams from 10 μg/L As(III) exposure group (Fig. 2D), while almost no changes were observed in other groups (Fig. 2E). In general, the transcriptional response of *GSTΩ 1* and *GSTΩ 2* to As(III) stress was more sensitive than to As(V) stress, and *GSTΩ 1* showed a more sensitive response to

inorganic arsenic exposure than *GSTΩ 2* at a transcriptional level.

3.5. Sequence analysis, recombinant protein expression and protein purification

The complete cDNA sequences of *GSTΩ 1* and *GSTΩ 2* have been deposited in the GenBank database under accession no. ADI44318 and AEW46328, respectively. The predicted amino acid sequences of both GSTΩ 1 and GSTΩ 2 exhibited strong identification with glutathione S-transferases from other mollusks. For example, GSTΩ 1 shared 46% similarity with glutathione S-transferase from *Haliotis madaka* (ALU63761), while GSTΩ 2 was mostly homologous with glutathione S-transferase from *Crassostrea virginica* (XP_022346075, 48% similarity). These two GSTΩs shared a 47% similarity with each other (Supplementary material, Fig. S2).

The purified rGSTΩ 1 and rGSTΩ 2 peptides were, respectively, represented as one distinct band (Supplementary material, Fig. S3, lane 4 and lane 9), with approximate molecular weights of 28 kDa and 25 kDa, respectively, which was consistent with the molecular weights deduced from the nucleotide sequences of GSTΩ genes. The concentrations of the purified rGSTΩ 1 and rGSTΩ 2 were 0.169 mg/mL and 0.293 mg/mL, respectively.

3.6. Immunoblotting and immunohistochemistry

The immunoblot analysis of the recombinant proteins produced only one single band, respectively, on the PVDF membranes that corresponded in size to purified rGSTΩ 1 and rGSTΩ 2 (Fig. S3, lane 5 and lane 10), indicating the specificity of the corresponding antibody.

The distribution and localization of GSTΩ 1 and GSTΩ 2 in the gills and digestive glands are illustrated in Fig. 3A and B. No or less positive staining was detected in the negative controls of the different tissues. The GSTΩ proteins were mainly expressed in the epithelial cells of gill filaments and hemocytes in gill cavities (Fig. 3A). In the digestive glands, intense staining was mainly exhibited in the columnar epithelial cells lining the lumen of the digestive tubules. Up-regulation of GSTΩ 1 and GSTΩ 2 expression was observed after 100 μg/L As(III) and 100 μg/L As(V) exposure compared to the corresponding controls (Fig. 3B). When the immunoreactivity of GSTΩ 1 and GSTΩ 2 was assessed through a western blot analysis in gills and digestive glands, two deepened bands were detected after arsenic exposure compared to the corresponding controls (Fig. 3C and D). Therefore, the expressions of GSTΩ proteins were up-regulated for arsenic detoxification under inorganic arsenic stress.

3.7. Effect of temperature, the substrate concentrations and pH on arsenate reductase activities of recombinant GSTΩs

As shown in Fig. 4A, the highest activities of recombinant GSTΩ 1 and GSTΩ 2 were both observed at 37 °C (pH 7.5). The arsenate reductase activities of rGSTΩ 1 and rGSTΩ 2 increased with increasing As(V) concentration and reached a peak at a concentration of 60 mM (Fig. 4B). Moreover, the arsenate reductase activities of rGSTΩs were higher at pH 4 and 5 compared to other pH values (Fig. 4C). In addition, the recombinant GSTΩ 1 possessed higher arsenate reductase activity than GSTΩ 2.

3.8. GSTΩ 1 and GSTΩ 2 complement arsenate-sensitive phenotype in E. Coli AW3110

As illustrated in Fig. 5A and D, in the LB medium containing 0 μM As(III) and As(V), the growth rate of the strains bearing empty plasmid and recombinant plasmids was apparently slower than that of *E. coli* AW3110 (DE3) (ΔarsRBC). For comparison, after a 16-hour incubation, the OD600 of *E. coli* AW3110 (ΔarsRBC) bearing recombinant plasmids pET-28a-GSTΩ 1 and pET-28a-GSTΩ 2 was 0.48 and 0.40, respectively,
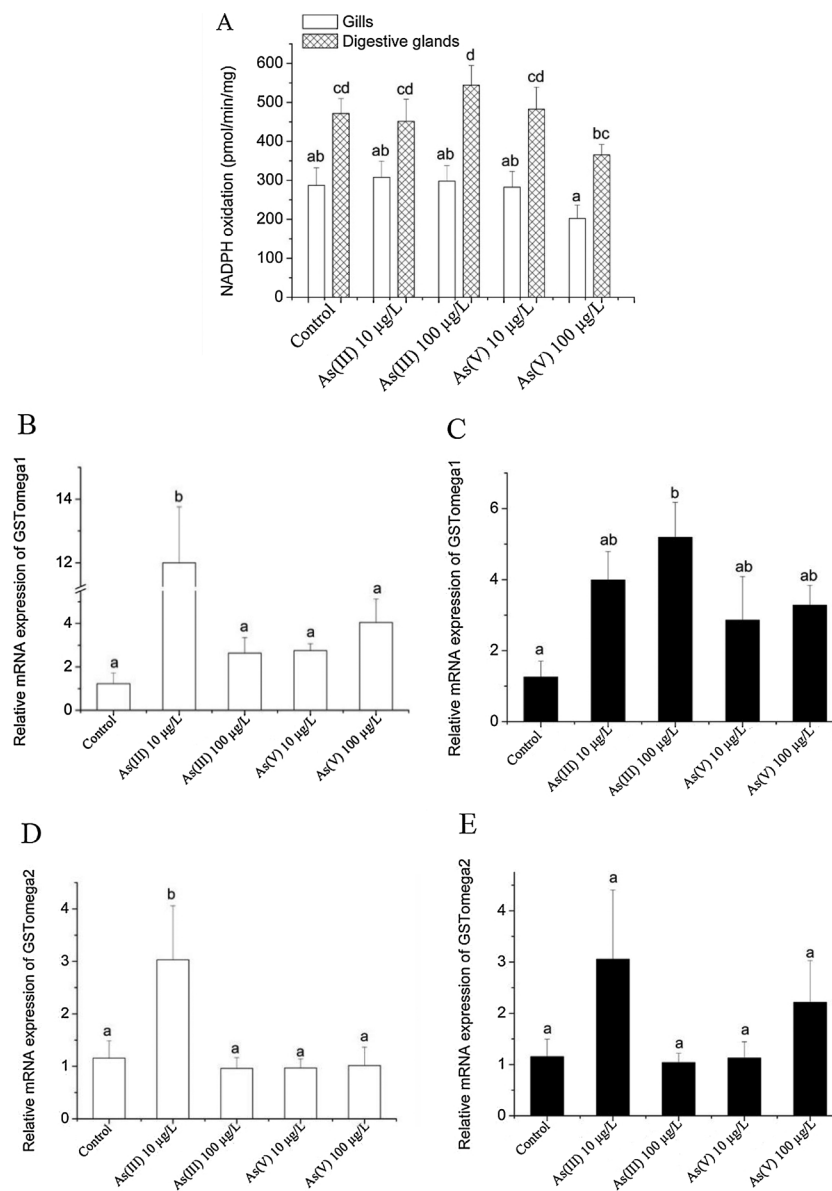
Fig. 2. Arsenate reductase activities and relative mRNA expression of GSTΩs in tissues.
(A) Arsenate reductase activities of GSTΩs in tissues after arsenic exposure ($n = 6$). Relative mRNA expression profiles of GSTΩ 1 and GSTΩ 2 genes in gills and digestive glands after arsenic exposure. (B) GSTΩ 1 in gills; **C.** GSTΩ 1 in digestive glands; (D) GSTΩ 2 in gills; (E) GSTΩ 2 in digestive glands. The values are shown as means ± SE ($n = 4$–6). Different letters indicate significant differences between the treatments ($P < 0.05$).

but the OD600 of *E. coli* AW3110 (Δ*arsRBC*) was 0.79. However, the expressions of GSTΩ 1 and GSTΩ 2 allowed for better growth than *E. coli* AW3110 (DE3) (Δ*arsRBC*) and *E. coli* AW3110 (DE3) (Δ*arsRBC*) bearing empty plasmid, even in concentrations of As(III) as high as 300 μM after more than a day of incubation (Fig. 5C), which indicated that GSTΩ 1 and GSTΩ 2 could offer resistance to As(III) in *E. coli* AW3110. However, the growth rate of *E. coli* AW3110 (DE3) (Δ*arsRBC*) bearing recombinant plasmids was still slower than *E. coli* AW3110 under elevated concentrations of As(V) exposure (Fig. 5E and F).

4. Discussion

4.1. Bioaccumulation and metabolism of arsenic

The arsenic levels in the tissues of marine organisms can widely vary from less than 1 ppm (dw) to more than several hundred ppm (dw). Concentrations often do not reflect the environmental levels of arsenic; thus, knowledge of the basal arsenic content in the tissues of

marine species is fundamental to realistically evaluate the significance of both the levels and the chemical speciation of this element (Fattorini et al., 2006; Meador et al., 2004). In this clam, the amount of total arsenic was up to 30.30 ± 5.70 μg/g dw in the gills and 19.56 ± 3.36 μg/g dw in the digestive glands, respectively, indicating a higher accumulation of arsenic in gills compared to the digestive glands. This was consistent with the fact that the arsenic accumulation was tissue-specific in aquatic organisms (Phillips and Depledge, 1986; Fattorini et al., 2004b). One explanation was that gills were directly in contact with arsenic in the seawater and mainly accumulated high concentrations of arsenic in the form of adsorption rather than absorption (Neff, 1997). However, the arsenic in the gills appeared easily discharged or transferred to other tissues such as digestive glands. It was also found that arsenic was depleted rapidly in gills but its concentration decreased slowly in digestive glands when the clams *Scrobicularia plana* were transplanted to a clean site (Langston, 1984). In this study, the total arsenic content in gills significantly increased after 100 μg/L As(III) exposure and 100 μg/L As(V) exposure, indicating
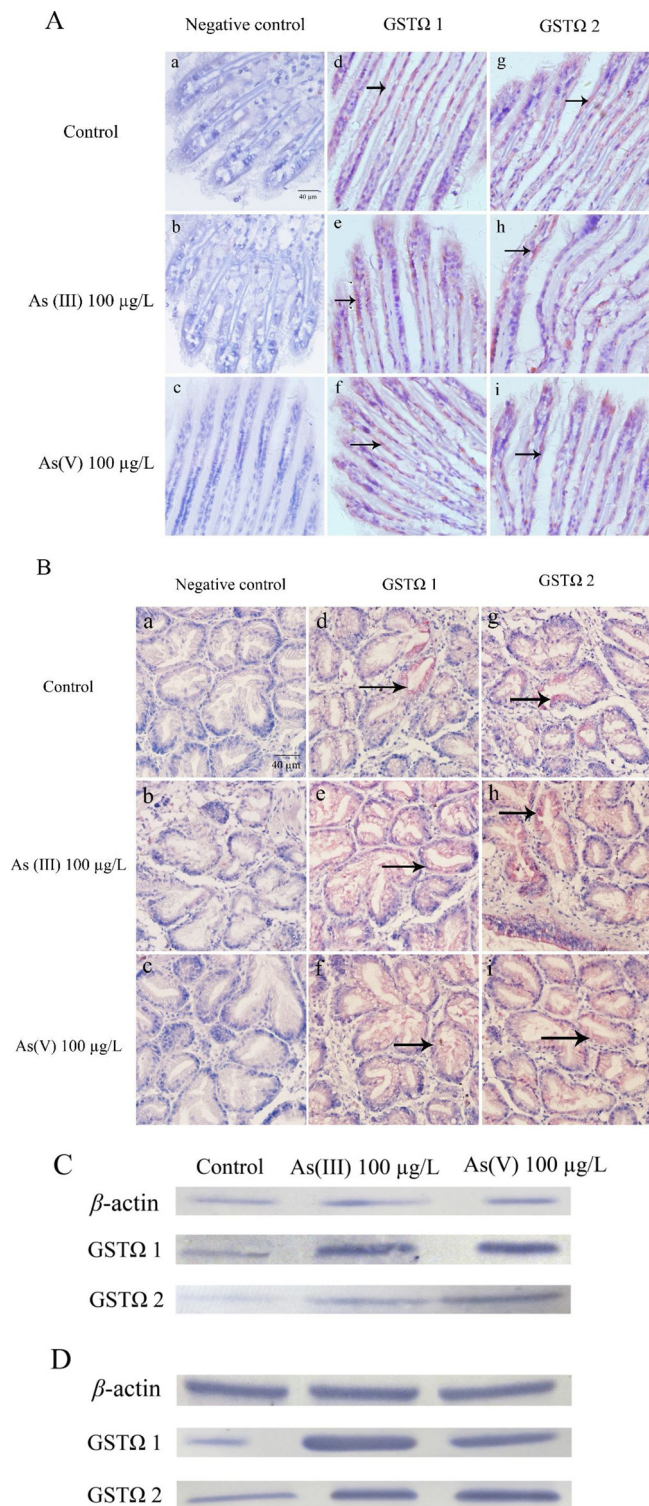
Fig. 3. Immunolocalization and western analysis of GSTΩs in tissues of *R. philippinarum*.
Immunolocalization of GSTΩ proteins in gills (A) and digestive glands (B). Negative controls of the control group (a), As(III) exposure group (b), As(V) exposure group (c). Localization of GSTΩ 1 of the control group (d), As(III) exposure group (e), As(V) exposure group (f). Localization of GSTΩ 2 of the control group (g), As(III) exposure group (h), As(V) exposure group (i). Positive signal was stained red (marked with arrow) and the other signals were stained blue. Western blot analysis of GSTΩs expressions in gills (C) and digestive glands (D). β-actin expressions were used to adjust for conformity of the soluble protein concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher bioavailability of As(III) compared to As(V) in the clam, which was similar to a previous study on the *Saccostrea cucullate* oyster (Zhang et al., 2015).

In marine organisms, arsenic generally existed in the form of organic arsenic species such as AsB, arsenocholine and arsenosugars (Fattorini et al., 2004a). Among them, AsB was the main organic form and the main end product of inorganic arsenic metabolism in many marine animals (Li et al., 2003a; Zhang et al., 2013). Our study also showed that the predominant form was AsB, with low concentrations of methylated and inorganic forms in this clam. As one of the metabolism intermediates of inorganic arsenic, MMA accounted for less than 6.78% in the gills and digestive glands of the clam. After inorganic arsenic exposure, the MMA content in digestive glands decreased significantly in the 100 μg/L As(V) exposure group, indicating that MMA can be quickly converted to other forms in marine invertebrates (Geiszinger et al., 2002; Zhang et al., 2015). Additionally, the DMA concentration in gills was almost twice that in digestive glands, suggesting that the conversion efficiency of inorganic arsenic was tissue specific in this clam. Therefore, digestive glands may play a vital role in arsenic metabolism and detoxification of *R. philippinarum*. Coincidentally, in vertebrates, the liver was shown to be the main site of arsenic methylation (Vahter, 2002).

4.2. Subcellular distribution

Organisms are able to control metal concentrations in certain tissues of their body to minimize damage from reactive forms of metals. Subcellular metal partitioning is fundamental for internal metal sequestration in different organs and tissues. Two major types of cellular sequestration involve the formation of distinct inclusion bodies and the binding of metals to heat-stable proteins. However, the compartmentalization or sequestration of metals by invertebrates is also dependent upon many factors, such as metal type, trophic transfer, and uptake routes (Cherian and Nordberg, 1983; Ng and Wang, 2005; Vijver et al., 2004). MTLPs are cysteine-rich proteins that have a significant capacity to bind metals and play an important role in the transportation and sequestration of intracellular metals (Klaassen et al., 1999). In marine animals, MTLPs were also the main subcellular distribution sites of trace metals in fish (Zhang et al., 2011, 2012a), oysters (Yu et al., 2013; Zhang et al., 2015) and polychaetes (Casado-Martinez et al., 2012). Therefore, the dominance of arsenic in MTLP fraction indicated that MTLPs probably played an important role in protecting the cells against arsenic toxicity in *R. philippinarum* as well.

From the standpoint of subcellular compartments, BDM probably played an important role in metal tolerance and resistance (Roesijadi, 1981). In this study, the clam *R. philippinarum* displayed a partitioning of arsenic to BDM to reduce arsenic toxicity. This finding was consistent with that reported in the polychaete *Arenicola marina,* which had most arsenic present in BDM after pollutant exposure (Casado-Martinez et al., 2012). Similarly, most Cd and Cu were also distributed in BDM in zebra mussels (Voets et al., 2009).

In digestive glands, the partitioning of arsenic to MSF decreased significantly after arsenic exposure ($P <$ 0.05). However, the partitioning of arsenic to cellular debris increased remarkably in the 10 μg/L As(III) and 10 μg/L As(V) exposure groups. Thus, cellular debris was also an important arsenic pool in marine animals (Zhang et al., 2011; Wang et al., 2011). The increasing distribution of arsenic in cellular debris might be due to the analogy of arsenic to phosphate, which functioned as the phospholipid components of cell membranes (Vijver et al., 2006). Similarly, less Zn was partitioned into MSF, and Zn in the cellular debris increased accordingly in *Daphnia magna* after Zn exposure (Wang and Guan, 2010).
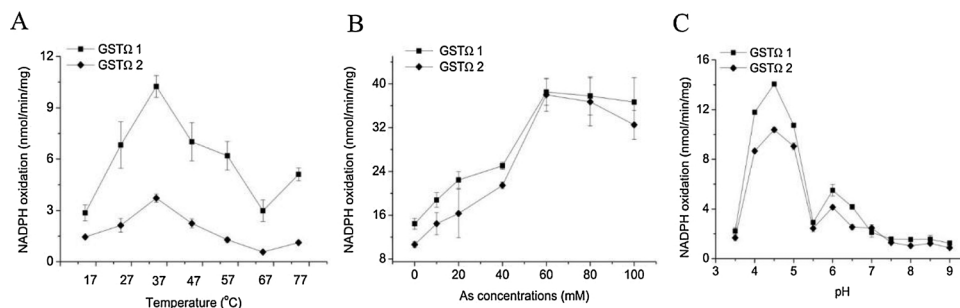
Fig. 4. Effect of temperature (A), As(V) concentrations (B) and pHs (C) on arsenate reductase activities of GSTΩ proteins. Arsenate reductase activities are shown as nmol NADPH/min/mg protein. The values were shown as means ± S.E. ($n = 3$).

4.3. Arsenate reductase activities, relative mRNA expressions and tissue distribution of GSTΩs in R. philippinarum

To date, little is known about the variation of GSTΩ activity following exposure to arsenic in marine animals (Ventura-Lima et al., 2011a). Our study found that arsenate reductase activities of GSTΩ increased dramatically in several exposure groups, indicating that GSTΩ might play a role in the metabolism and detoxification of arsenic. The subsequent decrease might be due to the aggravated tissue damage (data not shown) which interfered with the normal physiological functions and metabolism of arsenic. However, GSTΩ activities were restrained in the polychaete *Laeonereis acuta* after arsenite exposure but not significantly changed after arsenate exposure for 7 days (Ventura-Lima et al., 2011b). The differences might be related to the interspecific difference and exposure time.

The role of GSTΩ in arsenic detoxification was further investigated at the transcript level. It was found that the transcripts of *GSTΩ 1* and *GSTΩ 2* were moderately up-regulated after arsenic exposure,

suggesting a secondary role in As detoxification process. Similarly, the expression of two genes encoding GSTΩs in the abalone *Haliotis discus discus* also significantly increased after several trace metal stimulations (Wan et al., 2009). Additionally, *GSTΩ 1* was more responsive to arsenic exposure than *GSTΩ 2*. In disk abalone, *HdGSTO1* also exhibited a higher response than *HdGSTO2* after trace metal exposure (Wan et al., 2009).

Our results demonstrated that the transcription of the two *GSTΩ* genes were more sensitive to As(III) than As(V), which might be related to the higher bioavailability and toxicity of As(III) compared to As(V). Furthermore, these two genes displayed higher stress responses in digestive glands than in gills, suggesting that digestive glands might play a vital role in arsenic metabolism (Langston, 1984; Zhang et al., 2015). *GSTΩ 1* had been shown to be predominantly expressed in digestive glands (Zhang et al., 2012b). A high expression level of GSTs was also detected in digestive glands of the oyster *Crassostrea gigas* (Boutet et al., 2004). In vertebrates, liver was also the important expression site of GSH and GST that was involved in the metabolism of arsenic (Thomas,
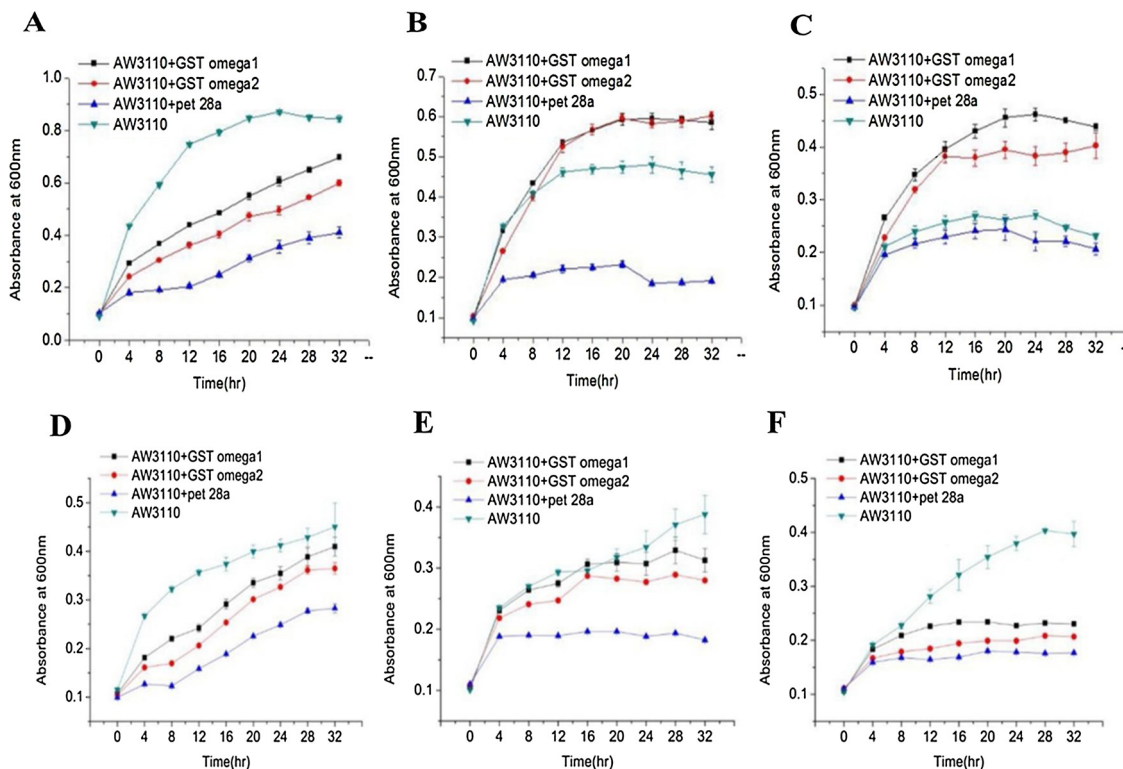

Fig. 5. Growth curve of *E. coli* with different concentrations of arsenic in the medium. A: 0 μM As(III), B: 100 μM As(III), C: 300 μM As(III), D: 0 μM As(V), E: 100 μM As(V), F: 300 μM As(V). Fig. 5 compiled data on the growth behavior of four kind strains, *E. coli* AW3110 (DE3) (*ΔarsRBC*), *E. coli* AW3110 (DE3) (*ΔarsRBC*) transformed with empty vector pET-28a, pET-28a-GSTΩ 1 and pET-28a-GSTΩ 2 at different concentrations of As(III) and As(V). The values were shown as means ± SE ($n = 3$).

2007). Therefore, GSTs might play a vital role in arsenic detoxification and metabolism in digestive glands.

An immunolocalization analysis showed that positive staining primarily appeared in the epithelial cells of the gill filaments, the hemocytes in gill cavities and the columnar epithelial cells lining the lumen of digestive tubules. There was clear immunoreactivity of GSTΩ 1 and GSTΩ 2 in digestive glands after 100 μg/L As(III) and 100 μg/L As(V) exposure. In humans, the gene encoding GSTΩ1-1 was also abundantly expressed in the liver, which was the main detoxification organ of pollutants (Yin et al., 2001). Additionally, a western blot analysis demonstrated the up-regulations of GSTΩ proteins in experimental groups compared to the control groups, indicating that the modulation of GSTΩ expressions was important for arsenic detoxification in this clam.

4.4. Effect of temperature, substrate concentrations and pH values on arsenate reductase activities of recombinant GSTΩs

The optimal pH and temperature for the arsenate reductase activities of GSTΩs were between 4–5 and 37 °C, respectively, while the arsenate reductase All0195 kept high activities at pH 7.5 and temperatures from 25 °C to 55 °C, showing high thermal stability (Pandey et al., 2013a). With the increase of substrate concentrations, the activities of GSTΩs increased until As(V) concentrations were up to 60 mM, complying with saturation kinetics. The Lineweaver-Burk Plot analysis revealed that the correlation coefficient was less than 0.9, indicating that the kinetics of GSTΩs were not in conformity with the Michaelis-Menten equation, which was similar to those of the arsenate reductase SynArsC in *Synechocystis* sp. PCC6803 (Li et al., 2003b). However, the activities of arsenate reductase from *Staphylococcus aureus* (Ji et al., 1994), rabbits (Zakharyan and Aposhian, 1999) and humans (Radabaugh et al., 2002) followed the Michaelis-Menten saturation kinetics. Thus, the arsenate reductase activity in different organisms might be affected by temperature and pH, and the kinetics also varied between different organisms.

4.5. GSTΩ 1 and GSTΩ 2 complement arsenate-sensitive phenotype in E. coli AW3110

The function of GSTΩ 1 and GSTΩ 2 was further verified by complementation assays in *E. coli* AW3110 (DE3) (ΔarsRBC). Our study showed that insertion expression vectors into *E. coli* AW3110 (DE3) (ΔarsRBC) might have reduced the growth rate of the bacteria. Moreover, both *E. coli* AW3110 (ΔarsRBC) following transformation with the conversion of recombinant plasmid pET-28a-GSTΩ 1 and pET-28a-GSTΩ 2 grew faster than *E. coli* AW3110 (DE3) (ΔarsRBC) in the 100 μM and 300 μM As(III) exposure groups. Hence, the GSTΩs can significantly enhance the arsenite tolerance of *E. coli* AW3110 (DE3) (ΔarsRBC), and GSTΩs were suggestive of the enzyme's significant role in arsenic detoxification. Interestingly, by contrast, the *GSTΩ* genes did not confer resistance to As(V) exposure, possibly due to the low expression of arsenate reductase *in vivo* and the preference for arsenic storage or segregation in the form of As(V). Above all, GSTΩs were presumed to mainly facilitate the combination of As(III) with GSH or other thiol-containing compounds, which was beneficial to As(III) discharge in this clam (Kumagai and Sumi, 2007).

In conclusion, the bioaccumulation and metabolism of inorganic arsenic and the possible function of GSTΩ proteins in detoxification were investigated in *R. philippinarum*. Inorganic arsenic was apt to accumulate in digestive glands, where the inorganic arsenic can be effectively converted into organic forms. BDM was found to be the main subcellular distribution site for arsenic. The mRNA and protein expressions of two *GSTΩ* genes were up-regulated after arsenic exposure. Additionally, GSTΩs show arsenate reductase activity and probably promote the combination of arsenite with thiol-containing compounds. Above all, GSTΩs make a significant contribution to As detoxification among the range of GSTs even though its comparative weight cannot be

fully ascertained. The roles of other GSTs in other marine organisms still need to be investigated in future studies.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2018.08.016.

References

Akter, K.F., Owens, G., Davey, D.E., Naidu, R., 2005. Arsenic speciation and toxicity in biological systems. Rev. Environ. Contam. Toxicol. 184, 97–149.

Aposhian, H.V., Zakharyan, R.A., Avram, M.D., Sampayo-Reyes, A., Wollenberg, M.L., 2004. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. Toxicol. Appl. Pharm. 198 (3), 327–335.

Boutet, I., Tanguy, A., Moraga, D., 2004. Characterisation and expression of four mRNA sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the Pacific oyster *Crassostrea gigas* exposed to hydrocarbons and pesticides. Mar. Biol. 146 (1), 53–64.

Casado-Martinez, M.C., Duncan, E., Smith, B.D., Maher, W.A., Rainbow, P.S., 2012. Arsenic toxicity in a sediment-dwelling polychaete: detoxification and arsenic metabolism. Ecotoxicology 21 (2), 576–590.

Cherian, M.G., Nordberg, M., 1983. Cellular adaptation in metal toxicology and metallothionein. Toxicology 28, 1–5.

Chowdhury, U.K., Zakharyan, R.A., Hernandez, A., Avram, M.D., Kopplin, M.J., Aposhian, H.V., 2006. Glutathione-S-transferase-omega [MMA(V) reductase] knockout mice: enzyme and arsenic species concentrations in tissues after arsenate administration. Toxicol. Appl. Pharmacol. 216 (3), 446–457.

Fattorini, D., Alonso-Hernandez, C.M., Diaz-Asencio, M., Munoz-Caravaca, A., Pannacciulli, F.G., Tangherlini, M., Regoli, F., 2004a. Chemical speciation of arsenic in different marine organisms: importance in monitoring studies. Mar. Environ. Res. 58 (2–5), 845–850.

Fattorini, D., Bocchetti, R., Bompadre, S., Regoli, F., 2004b. Total content and chemical speciation of arsenic in the polychaete *Sabella spallanzanii*. Mar. Environ. Res. 58 (2), 839–843.

Fattorini, D., Notti, A., Regoli, F., 2006. Characterization of arsenic content in marine organisms from temperate, tropical, and polar environments. Chem. Ecol. 22 (5), 405–414.

Francesconi, K.A., Edmonds, J.S., 1996. Arsenic and marine organisms. Adv. Inorg. Chem. 44, 147–189.

Gao, X., Zhou, F., Chen, C.T., 2014. Pollution status of the Bohai Sea: an overview of the environmental quality assessment related trace metals. Environ. Int. 62, 12–30.

Geiszinger, A.E., Goessler, W., Francesconi, K.A., 2002. The marine polychaete *Arenicola marina*: its unusual arsenic compound pattern and its uptake of arsenate from seawater. Mar. Environ. Res. 53 (1), 37–50.

Harju, T., Mazur, W., Merikallio, H., Soini, Y., Kinnula, V.L., 2008. Glutathione-S-transferases in lung and sputum specimens, effects of smoking and COPD severity. Respir. Res. 9, 80.

Ji, G., Garber, E.A., Armes, L.G., Chen, C.-M., Fuchs, J.A., Silver, S., 1994. Arsenate reductase of *Staphylococcus aureus* plasmid pI258. Biochemistry 33 (23), 7294–7299.

Jomova, K., Jenisova, Z., Feszterova, M., Baros, S., Liska, J., Hudecova, D., Rhodes, C.J., Valko, M., 2011. Arsenic: toxicity, oxidative stress and human disease. J. Appl. Toxicol. 31 (2), 95–107.

Katano, S., Matsuo, Y., Hanaoka, K., 2003. Arsenic compounds accumulated in pearl oyster *Pinctada fucata*. Chemosphere 53 (3), 245–251.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmacol. 39 (1), 267–294.

Kumagai, Y., Sumi, D., 2007. Arsenic: signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. Annu. Rev. Pharmacol. 47, 243–262.

Langston, W., 1984. Availability of arsenic to estuarine and marine organisms: a field and laboratory evaluation. Mar. Biol. 80 (2), 143–154.

Li, W., Wei, C., Zhang, C., Van Hulle, M., Cornelis, R., Zhang, X., 2003a. A survey of arsenic species in Chinese seafood. Food Chem. Toxicol. 41 (8), 1103–1110.

Li, R., Haile, J.D., Kennelly, P.J., 2003b. An arsenate reductase from *Synechocystis* sp. strain PCC 6803 exhibits a novel combination of catalytic characteristics. J. Bacteriol.

185 (23), 6780–6789.

Liu, X., Zhang, L., You, L., Yu, J., Cong, M., Wang, Q., Li, F., Li, L., Zhao, J., Li, C., Wu, H., 2011. Assessment of clam *Ruditapes philippinarum* as heavy metal bioindicators using NMR-based metabolomics. Clean – Soil Air Water 39 (8), 759–766.

Luo, W., Lu, Y., Wang, T., Hu, W., Jiao, W., Naile, J.E., Khim, J.S., Giesy, J.P., 2010. Ecological risk assessment of arsenic and metals in sediments of coastal areas of northern Bohai and Yellow Seas, China. Ambio 39 (5–6), 367–375.

Meador, J.P., Ernest, D.W., Kagley, A., 2004. Bioaccumulation of arsenic in marine fish and invertebrates from Alaska and California. Arch. Environ. Contam. Toxicol. 47 (2), 223–233.

Neff, J.M., 1997. Ecotoxicology of arsenic in the marine environment. Environ. Toxicol. Chem. 16 (5), 917–927.

Ng, T.Y.T., Wang, W.X., 2005. Dynamics of metal subcellular distribution and its relationship with metal uptake in marine mussels. Environ. Toxicol. Chem. 24 (9), 2365–2372.

Onsanit, S., Ke, C., Wang, X., Wang, K.J., Wang, W.X., 2010. Trace elements in two marine fish cultured in fish cages in Fujian province, China. Environ. Pollut. 158 (5), 1334–1342.

Pandey, S., Shrivastava, A.K., Singh, V.K., Rai, R., Singh, P.K., Rai, S., Rai, L.C., 2013a. A new arsenate reductase involved in arsenic detoxification in *Anabaena* sp. PCC7120. Funct. Integr. Genomic 13 (1), 43–55.

Phillips, D., Depledge, M., 1986. Distribution of inorganic and total arsenic in tissues of the marine gastropod *Hemifusus ternatanus*. Mar. Ecol.-Prog. Ser. 34 (3), 261–266.

Qin, J., Rosen, B.P., Zhang, Y., Wang, G., Franke, S., Rensing, C., 2006. Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase. P. Natl. Acad. Sci. U. S. A. 103 (7), 2075–2080.

Qin, J., Lehr, C.R., Yuan, C., Le, X.C., McDermott, T.R., Rosen, B.P., 2009. Biotransformation of arsenic by a Yellowstone thermoacidophilic eukaryotic alga. Proc. Natl. Acad. Sci. U. S. A. 106 (13), 5213–5217.

Radabaugh, T.R., Sampayo-Reyes, A., Zakharyan, R.A., Aposhian, H.V., 2002. Arsenate reductase II. Purine nucleoside phosphorylase in the presence of dihydrolipoic acid is a route for reduction of arsenate to arsenite in mammalian systems. Chem. Res. Toxicol. 15 (5), 692–698.

Rahman, M.A., Hassler, C., 2014. Is arsenic biotransformation a detoxification mechanism for microorganisms? Aquat. Toxicol. 146, 212–219.

Roesijadi, G., 1981. The significance of low molecular weight, metallothionein-like proteins in marine invertebrates: current status. Mar. Environ. Res. 4 (3), 167–179.

Sampayo-Reyes, A., Zakharyan, R.A., 2006. Inhibition of human glutathione S-transferase omega by tocopherol succinate. Biomed. Pharmacother. 60 (5), 238–244.

Thomas, D.J., 2007. Molecular processes in cellular arsenic metabolism. Toxicol. Appl. Pharm. 222 (3), 365–373.

Thomas, D.J., Nava, G.M., Cai, S.Y., Boyer, J.L., Hernandez-Zavala, A., Gaskins, H.R., 2010. Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals in the invertebrate chordate *Ciona intestinalis*. Toxicol. Sci. 113 (1), 70–76.

Vahter, M., 2002. Mechanisms of arsenic biotransformation. Toxicology 181, 211–217.

Ventura-Lima, J., Fattorini, D., Regoli, F., Monserrat, J.M., 2009. Effects of different inorganic arsenic species in *Cyprinus carpio* (Cyprinidae) tissues after short-time exposure: bioaccumulation, biotransformation and biological responses. Environ. Pollut. 157 (12), 3479–3484.

Ventura-Lima, J., Bogo, M.R., Monserrat, J.M., 2011a. Arsenic toxicity in mammals and aquatic animals: a comparative biochemical approach. Ecotox. Environ. Saf. 74 (3), 211–218.

Ventura-Lima, J., Ramos, P.B., Fattorini, D., Regoli, F., Ferraz, L., de Carvalho, L.M., Monserrat, J.M., 2011b. Accumulation, biotransformation, and biochemical responses after exposure to arsenite and arsenate in the estuarine polychaete *Laeonereis acuta* (Nereididae). Environ. Sci. Pollut. Res. Int. 18 (8), 1270–1278.

Vijver, M.G., van Gestel, C.A., Lanno, R.P., van Straalen, N.M., Peijnenburg, W.J., 2004. Internal metal sequestration and its ecotoxicological relevance: a review. Environ. Sci. Technol. 38 (18), 4705–4712.

Vijver, M.G., van Gestel, C.A., van Straalen, N.M., Lanno, R.P., Peijnenburg, W.J., 2006. Biological significance of metals partitioned to subcellular fractions within

earthworms (*Aporrectodea caliginosa*). Environ. Toxicol. Chem. 25 (3), 807–814.

Voets, J., Redeker, E.S., Blust, R., Bervoets, L., 2009. Differences in metal sequestration between zebra mussels from clean and polluted field locations. Aquat. Toxicol. 93 (1), 53–60.

Wallace, W.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM). Mar. Ecol.-Prog. Ser. 257, 125–137.

Wan, Q., Whang, I., Lee, J.S., Lee, J., 2009. Novel omega glutathione S-transferases in disk abalone: characterization and protective roles against environmental stress. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 150 (4), 558–568.

Wang, W.X., Guan, R., 2010. Subcellular distribution of zinc in *Daphnia magna* and implication for toxicity. Environ. Toxicol. Chem. 29 (8), 1841–1848.

Wang, W.X., Yang, Y., Guo, X., He, M., Guo, F., Ke, C., 2011. Copper and zinc contamination in oysters: subcellular distribution and detoxification. Environ. Toxicol. Chem. 30 (8), 1767–1774.

Wang, Q., Zhang, L., Zhao, J., You, L., Wu, H., 2012. Two goose-type lysozymes in *Mytilus galloprovincialis*: possible function diversification and adaptive evolution. PLoS One 7 (9), e45148.

Waring, J., Maher, W., 2005. Arsenic bioaccumulation and species in marine polychaeta. Appl. Organomet. Chem. 19 (8), 917–929.

Xu, L., Wang, T., Ni, K., Liu, S., Wang, P., Xie, S., Meng, J., Zheng, X., Lu, Y., 2013. Metals contamination along the watershed and estuarine areas of southern Bohai Sea, China. Mar. Pollut. Bull. 74 (1), 453–463.

Yang, J., Wang, W., Wei, X., Qiu, L., Wang, L., Zhang, H., Song, L., 2010. Peptidoglycan recognition protein of *Chlamys farreri* (CfPGRP-S1) mediates immune defenses against bacterial infection. Dev. Comp. Immunol. 34 (12), 1300–1307.

Yin, Z.-L., Dahlstrom, J.E., Le Couteur, D.G., Board, P.G., 2001. Immunohistochemistry of omega class glutathione S-transferase in human tissues. J. Histochem. Cytochem. 49 (8), 983–987.

Yin, X.X., Chen, J., Qin, J., Sun, G.X., Rosen, B.P., Zhu, Y.G., 2011. Biotransformation and volatilization of arsenic by three photosynthetic cyanobacteria. Plant Physiol. 156 (3), 1631–1638.

Yu, X.J., Pan, K., Liu, F., Yan, Y., Wang, W.X., 2013. Spatial variation and subcellular binding of metals in oysters from a large estuary in China. Mar. Pollut. Bull. 70 (1–2), 274–280.

Zakharyan, R.A., Aposhian, H.V., 1999. Enzymatic reduction of arsenic compounds in mammalian systems: the rate-limiting enzyme of rabbit liver arsenic biotransformation is MMAV reductase. Chem. Res. Toxicol. 12 (12), 1278–1283.

Zakharyan, R.A., Sampayo-Reyes, A., Healy, S.M., Tsaprailis, G., Board, P.G., Liebler, D.C., Aposhian, H.V., 2001. Human monomethylarsonic acid (MMAV) reductase is a member of the glutathione-S-transferase superfamily. Chem. Res. Toxicol. 14 (8), 1051–1057.

Zhang, W., Wang, W.-X., 2012. Large-scale spatial and interspecies differences in trace elements and stable isotopes in marine wild fish from Chinese waters. J. Hazard. Mater. 215–216, 65–74.

Zhang, W., Huang, L., Wang, W.X., 2011. Arsenic bioaccumulation in a marine juvenile fish *Terapon jarbua*. Aquat. Toxicol. 105 (3-4), 582–588.

Zhang, W., Huang, L., Wang, W.X., 2012a. Biotransformation and detoxification of inorganic arsenic in a marine juvenile fish *Terapon jarbua* after waterborne and dietborne exposure. J. Hazard. Mater. 221–222, 162–169.

Zhang, L., Qiu, L., Wu, H., Liu, X., You, L., Pei, D., Chen, L., Wang, Q., Zhao, J., 2012b. Expression profiles of seven glutathione S-transferase (GST) genes from *Venerupis philippinarum* exposed to heavy metals and benzo[a]pyrene. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155 (3), 517–527.

Zhang, W., Wang, W.X., Zhang, L., 2013. Arsenic speciation and spatial and interspecies differences of metal concentrations in mollusks and crustaceans from a South China estuary. Ecotoxicology 22 (4), 671–682.

Zhang, W., Guo, Z., Zhou, Y., Liu, H., Zhang, L., 2015. Biotransformation and detoxification of inorganic arsenic in Bombay oyster Saccostrea cucullata. Aquat. Toxicol. 158, 33–40.