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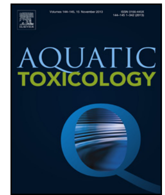

Biotransformation and detoxification of inorganic arsenic in Bombay oyster *Saccostrea cucullata*

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ABSTRACT

Arsenic (As) exists as the toxic inorganic forms in marine water and sediment, while marine oysters usually accumulate high As contents mostly as the less toxic organic forms. It has not yet been clear that how As is biotransformed in marine oysters. This study therefore investigated the biotransformation and detoxification of two inorganic As forms (As(III) and As(V)) in Bombay oyster *Saccostrea cucullata* after waterborne exposures for 30 days. Seven treatments of dissolved As exposure (clean seawater, 1, 5, 20 mg/L As(III), and 1, 5, 20 mg/L As(V)) were performed. Body As concentration increased significantly after all As exposure treatments except 1 mg/L As(V). Total As, As(III), and As(V) concentration were positive correlated with glutathione-S-transferases (GST) activities, suggesting GST might play an important role in the As biotransformation and detoxification process. Organic As species were predominant in control and the low As exposed oysters, whereas a large fraction of As was remained as the inorganic forms in the high As exposed oysters, suggesting As could be biotransformed efficiently in the oysters in clean or light contaminated environment. The results of As speciation demonstrated the As biotransformation in the oysters included As(V) reduction, methylation to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), and subsequent conversion to arsenobetaine (AsB). More As was distributed in the subcellular metallothionein-like proteins fraction (MTLP) functioning sequestration and detoxification in the inorganic As exposed oysters, suggesting it was also a strategy for oysters against As stress. In summary, this study elucidated that marine oysters had high ability to accumulate, biotransform, and detoxify inorganic As.

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1. Introduction

Arsenic (As) is a highly toxic environmental pollutant, ranking first in the 'Superfund List of Hazardous Substances' (ATSDR, 2013). As pollution has become a serious environmental problem in many Asian areas. For example, As concentrations was up to 5 mg/L in the contaminated groundwater due to mining and dredged alluvium in Thailand (Nordstrom, 2002). The total As in the sediment of the Pearl River in Guangzhou, China ranged from 16.7 to 33.4 mg/kg, as the outcome of the long-term intensive industrial and urban activities (Wang et al., 2010).

Inorganic As compounds are the major forms in seawater and sediments, while organic As compounds like arsenobetaine (AsB), arsenocholine (AsC) and arsenosugars (AsS) are generally predominated in marine organisms (Fattorini et al., 2004, 2006). The toxicity of As to organisms depends on its concentration and speciation (Sharma and Sohn, 2009; Jomova et al., 2011). Inorganic As displays extreme toxicity and is a class A carcinogen (Sabbioni et al., 1991), while organic As – such as AsB, AsC, and several AsS – are considered to be less toxic. Marine organisms accumulate, retain, and transform As species inside their bodies when exposed to it through seawater (Hasegawa et al., 2001; Suhendrayatna et al., 2001). To date, there have been numerous studies quantified the biotransformation of As in eukaryotic alga (Qin et al., 2009), fungi (Su et al., 2011), lichens (Mrak et al., 2008; Pisani et al., 2011), polychaete (Geiszinger et al., 2002), and marine fish (Bagnyukova et al., 2007; Zhang et al., 2012). While little has been known about the biotransformation of different inorganic As forms in oyster.

Aquatic organisms have developed corresponding strategies to detoxify As, including (1) reduction of As(V) to As(III) followed by

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either excretion or complexation with glutathione and sequestration into vacuoles (e.g., *Saccharomyces cerevisiae*, Rosen, 1999); (2) reduction of As(V) to As(III) followed by methylation to trimethylarsine (TMA(III)), that would leave the cell as a volatile gas (e.g., eukaryotic alga, Qin et al., 2009); and (3) the reduction of As(V) to As(III) followed by methylation to AsB or AsC (e.g., mollusks, Sörös et al., 2003; marine fish, Zhang et al., 2012). Thus, As biotransformation is one of the detoxification strategies. Moreover, in the biotransformation process, As(III)-thiol complexation occurs in S-rich proteins, and glutathione As(III)-thiol complexes are methylated to organoarsenic compounds (e.g. AsB) (Langdon et al., 2002). The measurement of glutathione S-transferase (GST) activities has been used to estimate the capability of a tissue to metabolize inorganic As (Aposhian et al., 2004; Sampayo-Reyes and Zakharyan, 2006; Bhattacharya and Bhattacharya, 2007).

Other than As biotransformation, our previous study demonstrated that metallothionein-like proteins (MTLPs) could also involve in As detoxification in a marine fish (Zhang et al., 2012). MTLPs are cysteine-rich metal-binding proteins, and numerous studies have verified their roles in the sequestration and detoxification of intracellular metals such as Cd, Zn and Hg for decades (Nordberg, 1998, 2009; Klaassen et al., 1999). It is interesting to known whether MTLPs is a universal strategy for As detoxification in different aquatic organisms.

Oysters are one of the most widespread bivalve species. They are great pollutant accumulators and good indicators of contamination (Marie et al., 2006; Valdez Domingos et al., 2007). Bioaccumulation and speciation of As in marine oyster have been studied in recent years (Vilanó and Rubio, 2001; Liu et al., 2007; Khokiattiwong et al., 2009; Zhang et al., 2013). Our previous study investigated the As contamination in oyster *Saccostrea cucullata* in the intertidal zone along the Zhanjiang coastal waters in China, and we found the total As concentrations were 4.22–13.2 mg/kg and the AsB constituted 83.0–95.1%, suggesting that oyster has high ability to accumulate and biotransform As (Zhang et al., 2013). To date, the studies about As speciation in the oysters just focused on the detection of the field samples, while few attempts have been made to investigate the mechanisms of inorganic As biotransformation in oysters.

Therefore, the objective of this work was to characterize the bioaccumulation, speciation, biotransformation, and detoxification of As in a common edible oyster, *S. cucullata*, following a series of long-term waterborne As exposures. We consequently analyzed As speciation to comprehend the mechanisms of As biotransformation and detoxification, measured GST activities to indicate the role of GST in As biotransformation and detoxification, and characterized the subcellular fates of As to demonstrate the detoxification strategies. Overall, we tried to link inorganic As exposure and its biotransformation and detoxification in oysters.

2. Materials and methods

2.1. Oyster and experimental design

Bombay oyster *S. cucullata* were obtained from a seafood market nearby the Pearl River Estuary, in Zhuhai, China, in October 2013. To minimize the effects of body size on the results of As concentrations, a uniform size of sample (4–5 cm) was selected. After transport to the lab, oysters were thoroughly brushed to remove mud and clean the shells, then they were maintained in artificial seawater (20 °C, 20‰) (composed by recrystallized sea salt, Landebao Com., China) and fed algae powders (proteins, 60–65%; carbohydrates, 15–20%; lipids, 5%; minerals, 5–8%; total As, 0.85 ± 0.03 μg/g) once a day under a light:dark cycle of 12:12 h in the lab. They were acclimated to the test conditions for 2 weeks prior to the exposure experiment.

The oysters were randomly separated and placed in aquaria filled with 5 L of artificial seawater. There were seven treatments of different dissolved As(III) and As(V) concentrations, clean seawater (1.2 ± 0.1 μg/L, or 16 nmol/L of the total As), 1 mg/L (13 μmol/L), 5 mg/L (67 μmol/L), 20 mg/L (267 μmol/L) As(III) and As(V)). Each treatment had two independent replicate aquariums with nine individual oysters. The exposure media were prepared by spiking an appropriate volume of 1000 mg/L stock solution (as $NaAsO_2$ and $Na_2HAsO_4{\cdot}7H_2O$) into artificial seawater. The oysters were exposed to dissolved As(III) and As(V) for 22 h and fed with algal powders for 2 h in clean artificial seawater to avoid any possible influence of the food on the dissolved As uptake every day. The exposure water was completely renewed every day after feeding. Seawater was sampled before and after each renewal process in experimental treatments in order to assess any potential decrease of the nominal concentrations. The As level in seawater kept as 90–96% of the nominal concentrations. The aquariums were checked frequently to remove the dead oysters during exposure period.

The live oysters were collected on the last days of the 1-month exposure, while oysters exposed to 5 and 20 mg/L As(III) were sampled in the first 2 weeks since few of them survived until the end of exposure due to As(III) toxicity. The oysters were collected prior to the feedings to avoid possible gut contamination and were then shucked and rinsed, wet-weighed, stored at −80 °C for the analyses of total As, As speciation, GST activities and subcellular fractionation.

2.2. Total arsenic concentrations

The frozen oysters in each treatment were freeze dried and homogenized for total As and As speciation analysis. About 0.02 g tissues were digested in 1 mL of concentrated HNO_3 (65%, analytical reagent grade, Fisher Scientific) for 2 h reaction at room temperature and 24 h reaction at 80 °C until the reagent was totally clear. The samples were diluted to 10 mL with double deionized water (Millipore). Blank samples were processed using the similar procedure. The samples were analyzed for total As using the inductively coupled plasma mass spectrometer (ICP–MS). The standard solution was prepared by serial dilution from a stock solution (National China Standard, National Institute of Metrology, China). The accuracy of our digestion method was testified by analyzing the standard reference material of 1566b-oyster tissue. The total As recovery rate of the reference material was 98.6%. The As concentrations in the oyster tissue were expressed as μg/g dry weight.

2.3. Arsenic speciation analysis

All solutions were prepared with ddH_2O. Stock standard solutions of As compounds were prepared from sodium arsenite ($NaAsO_2$) (Sigma, USA), sodium arsenate dibasic heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$) (Sigma, USA), sodium cacodylatetrihydrate ($C_2H_6AsNaO_2{\cdot}3H_2O$) (Sigma, USA), disodium methylarsenate ($CH_3AsO_3Na_2{\cdot}6H_2O$) (SUPELCO, USA), arsenobetaine ($(CH_3)_3As^+CH_2COO^-$) (Fluka, Sigma). Chemicals used in HPLC mobile phases (NH_4HCO_3 and KCl) and HCl and KOH employed for hydride generations were all obtained from Guangzhou Chemical Reagent Factory (China). KBH_4 was obtained from CNW Technologies GmbH, and $K_2S_2O_8$ used in the photo-oxidation reaction was purchased from Guangzhou Chemical Reagent Factory (China). The KBH_4 solution was not filtered before use, and was prepared daily. Samples were filtered through a 0.45 μm PTFE membrane (China). The standard solutions were stored in high density polypropylene containers at 4 °C. Analytical working standards were prepared daily by diluting the stock solutions with ddH_2O prior to analysis.

The freeze-dried oyster tissues were prepared for As speciation analysis using methanol/water (1:1 v/v) extraction as described

previously (Shibata and Morita, 1992; Lai et al., 2002). About 0.05 g of sample was accurately weighed and transferred into 10 mL centrifuge tubes with 5 mL of 50% methanol (50% MeOH in ddH_2O) solution. The mixtures were homogenized with a tissue homogenizer for 15 min, then centrifuged at 10,000 rpm for 10 min and the supernatant then poured into a 50 mL centrifuge tubes. This extraction process was repeated twice with the supernatant being added to the previous extract. The final extract (a combination of the two supernatants approximately 10 mL in total) was heated to 50 °C to evaporate the solvent until a volume of approximately 1 mL was reached. The concentrated samples were then diluted with ddH_2O to a volume of 4 mL. Samples were filtered through 0.45 μm syringe filters into 5 mL centrifuge tube in preparation for HPLC-UV-HG-AFS (AF-610D2 from Beifenruili Analytical Instrument Corp., Beijing, China) analysis. The extracted samples (40 μL) were injected into the chromatographic column used for As-species separation.

The extraction efficiencies and analysis methods were evaluated by the analysis of standard reference materials tuna fish (BCR-627, Institute for Reference Materials and Measurements, Geel, Belgium). BCR-627 tuna fish tissue (0.1 g) was used for AsB and DMA analyses. The BCR-627 reference material contained AsB 4.27 ± 0.23 μg/g (109% recovery of 3.90 ± 0.22 μg/g certified value, n = 6) and DMA 1.31 ± 0.31 μg/g (87% recovery of 1.5 ± 0.02 μg/g certified value, n = 6). Spikes were used to confirm the recovery of other As species detected during speciation analysis. In our study, As(III) recoveries were 79–96%, As(V) recoveries were 76–102%, and MMA recoveries were 87–92%.

2.4. Glutathione-S-transferases activities

Fresh tissues were washed with ice cold in 0.86% (w/v) NaCl solution. About 0.05 mg tissues were homogenized using a tissue homogenizer in 0.86% NaCl solution. The homogenate was centrifuged at 3500 rpm at 4 °C for 10 min. The supernatants were collected for GST enzyme activity assay. The protein concentration was measured by using a Bi Yuntian BCA assay kit (Haimen, China). GST was determined spectrophotometrically using commercially available GST activity kits based upon the GST-catalyzed reaction between glutathione (GSH) and the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

2.5. Subcellular arsenic distribution

The subcellular As fractionation in oysters was carried out according to a well-established protocol (Wallace et al., 2003; Pan and Wang, 2008). Five subcellular fractions were separated (cellular debris, metal-rich granules (MRG), organelles, heat-sensitive protein (HSP), and MTLPs). Briefly, the oyster tissues were homogenized with a tissue homogenizer in 20 mmol/L Tris–HCl buffer spiked with 2-mercaptoethanol (5 mM) and henylmethanesulfonyl fluoride (0.1 mM) (pH 7.4). The homogenized oyster tissues were firstly centrifuged at 1500 × g at 4 °C for 15 min. The separated pellets were digested in 4 mL of 1 mol/L NaOH at 80 °C for 10 min and centrifuged at 5000 × g for 10 min at 4 °C to separate the cellular debris and the MRG. The supernatant from the first centrifugation was further centrifuged at 100,000 × g at 4 °C for 1 h to separate the organelles and the cytosol. Afterward, the cytosol was heated at 80 °C for 10 min, ice-cooled for 1 h, and further centrifuged at 30,000 × g at 4 °C for 10 min to obtain the HSP and the MTLP. Afterward, the five fractions were digested in concentrated nitric acid (HNO_3, 65%) similarly as described above. Total As concentration in each fraction was measured by ICP–MS. The As subcellular distribution was defined as the percentage of As in each fraction.

2.6. Statistical analyses

Statistical analyses were performed using SPSS version 16.0. The differences of the corresponding values between the control and exposed groups were tested by one-way analysis of variance (ANOVA) followed by a Duncan test. A probability level (p-value) of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Arsenic bioaccumulation

After 30 days, mortalities of oysters were 11.1 ± 0.0%, 16.7 ± 7.8%, 22.2 ± 15.7%, 27.8 ± 7.8%, and 27.8 ± 7.8% in control, 1 mg/L As(III), 1 mg/L As(V), 5 mg/L As(V), and 20 mg/L As(V) exposure treatments, respectively. 100% mortality was received in 5 and 20 mg/L As(III) treatments after 15 days exposure.

All the As species and total As concentrations in the control oysters after 30-days exposure were similar to the ones before exposure. There were significant increases in As bioaccumulation in 1 mg/L As(III), 5 mg/L As(V), and 20 mg/L As(V) exposed oysters. The high As exposure concentrations (1 mg/L As(III), 5 mg/L and 20 mg/L As(V)) probably influenced the patterns of As(III) and As(V) bioaccumulation in the oysters. However, there were no significant increases in As bioaccumulation in 1 mg/L As(V) exposed oysters. Body As concentrations increased proportionally to the dissolved As concentrations in the As(V) exposure treatments. As bioaccumulation was higher in 1 mg/L As(III) exposure than 1 mg/L As(V) exposure, suggesting that As(III) may be more bioavailable than As(V) in oysters when facing high As exposure concentrations. Instead, As concentrations in 1 mg/L As(III) exposed oysters were comparable with that in 5 mg/L As(V) exposed ones (Fig. 1).

3.2. Biotransformation of arsenic

Table 1 and Fig. 2 present the concentrations of the five As species and their percentages in oyster tissues. AsB exhibited as a predominant As species, about 61.41%, 56.26%, 53.18%, 32.10% in control, 1 mg/L As(III), 1 mg/L As(V) and 5 mg/L As(V), respectively, while AsB exhibited only a small fraction (2.29%) in 20 mg/L As(V) exposure. DMA was about 15.27% in control. MMA was the smallest fraction (0.48–3.34%) in all the treatments. Interestingly, DMA and MMA levels increased significantly upon exposure to waterborne

Table 1
As speciation concentrations (μg/g, dry weight) in the oyster tissues after waterborne inorganic As exposure for 30 days. As(III), arsenite; As(V), arsenate; MMA, monomethylarsonate; DMA, dimethylarsinate; AsB, arsenobetaine. Data shown are mean ± SD (n = 3–6). Different letters indicate statistically significant difference between As exposure treatments.

	As(III)	As(V)	MMA	DMA	AsB
Control	0.477 ± 0.156^{a}	0.357 ± 0.038^{a}	0.130 ± 0.028^{a}	1.30 ± 0.276^{a}	5.12 ± 0.590^{a}
As(III) 1 mg/L	27.4 ± 1.84^{c}	7.14 ± 3.64^{b}	0.567 ± 0.258^{a}	0.855 ± 0.332^{a}	64.7 ± 0.538^{d}
As(V) 1 mg/L	3.29 ± 0.335^{b}	6.43 ± 0.167^{b}	0.353 ± 0.171^{a}	2.28 ± 0.090^{b}	21.2 ± 1.22^{c}
As(V) 5 mg/L	23.5 ± 2.87^{c}	28.3 ± 7.33^{c}	1.56 ± 0.046^{b}	10.2 ± 2.83^{c}	22.9 ± 3.21^{c}
As(V) 20 mg/L	111 ± 5.91^{d}	298 ± 65.6^{d}	15.7 ± 3.03^{c}	36.5 ± 1.17^{d}	10.5 ± 0.533^{b}

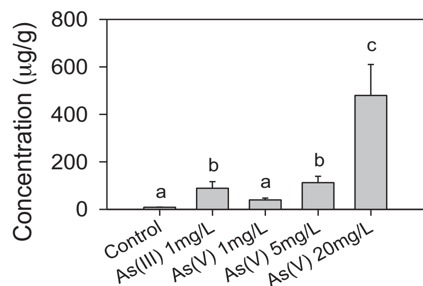

Fig. 1. Total As concentrations (μg/g, dry weight) in the oysters after waterborne inorganic As exposure for 30 days. Data are mean ± SD (n = 12–18). Different letters represent significant difference between treatments (p < 0.05).

As(V) 5 mg/L and As(V) 20 mg/L for 30 days, respectively, but was very low after As(III) waterborne exposures. Furthermore, one unknown As compound (possibly AsS) was found. Overall, organic As contributed more than half of the total As (57.46–78.25%) in all the treatments except 20 mg/L As(V) exposure, strongly indicating that oysters have high ability to biotransform inorganic As into organic forms.

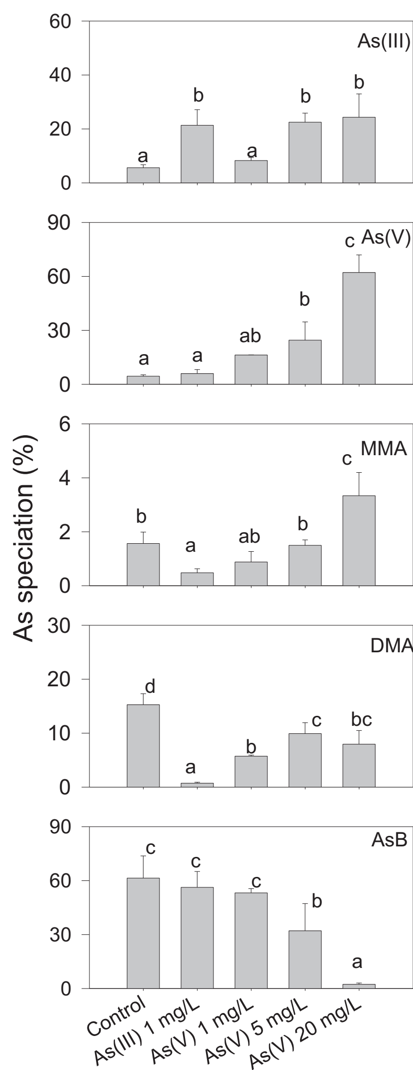

Fig. 2. The proportion of different As species (%) in the oysters after waterborne inorganic As exposure for 30 days. Data are mean ± SD (n = 6). As(III), arsenite; As(V), arsenate; MMA, monomethylarsonate; DMA, dimethylarsinate; AsB, arsenobetaine.

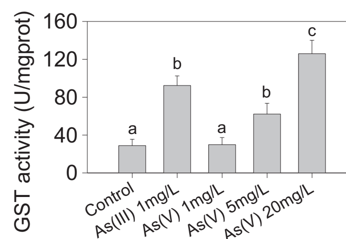

Fig. 3. Glutathione-S-transferase activity (U/mg protein) in the oysters after waterborne inorganic As exposure for 30 days. Data are mean ± SD (n = 6).

As(III) was about 23.96% in the 1 mg/L As(III) exposed oysters, As(V) was about 16.16%, 24.53%, and 62.12% in the 1 mg/L, 5 mg/L, and 20 mg/L As(V) exposed oysters, respectively. Inorganic As was a notable fraction under As(III) or As(V) exposure, and concentrations and proportions of As(V) and As(III) generally increased with the increasing As(V) exposure concentrations. Thus, the oysters had different biotransformation efficiencies in response to As stress, the high efficiencies were found when facing low As exposure concentrations, yet relatively low efficiencies were found when facing high As exposure concentrations. Therefore, the high As exposure concentrations (5 mg/L and 20 mg/L As(V)) probably influenced the patterns of As(III) and As(V) biotransformation in the oysters.

3.3. Glutathione-S-transferases activities

GST activities were enhanced significantly in 1 mg/L As(III), 5 mg/L and 20 mg/L As(V) exposed oysters (Fig. 3), however, GST activities were comparable in control and 1 mg/L As(V) treatments. GST activities increased proportionally to the dissolved As concentrations in As(V) exposure treatments. The GST activities were higher after 1 mg/L As(III) exposure than 1 mg/L As(V) exposure. The total As, As(III), As(V), and MMA concentrations in the oyster tissues were positive correlated with GST activities in waterborne As(V) exposure treatments after 30 days, suggesting that GST may play an important role in the As biotransformation process (Fig. 4).

3.4. Subcellular distribution

Fig. 5 shows the subcellular distribution of As in the oysters. In general, MTLP was the major binding site for As in the oyster tissues (58.1% in control, and 76.2–85.2% after As(III) and As(V) exposure). MTLP in 5 mg/L As(V) and 20 mg/L As(V) were significantly higher than 1 mg/L As(V), 1 mg/L As(III), and control treatment, suggesting more As distributed in the MTLP fraction when exposed to higher As(V) concentrations. Cellular debris was another important fraction (22.8% in control, 11.6% in 1 mg/L As(III), and 11.3% in 1 mg/L As(V)). Cellular debris in 5 mg/L and 20 mg/L As(V) were significantly lower than the other three treatments. Only a small fraction of As was bound with MRG (6.7% in control and 1.0–2.3% in As(III) and As(V) exposure), organelles (9.9% in control and 5.5–6.1% in As(III) and As(V) exposure), and HSP (5.1–6.2% in all the treatments). MRG, organelles, and cellular debris in control were significantly higher than in As exposure treatments.

Fig. 6 shows the correlations between the subcellular distribution of As and As bioaccumulation in the oysters in control and As(V) exposure treatments. Total As concentration was positive correlated with MTLP, but was negative correlated with MRG, organelles, and cellular debris, suggesting As bioaccumulation in the exposed oysters affected the distribution of subcellular level. More As was distributed in the MTLP when more As was bioaccumulated in the oysters.

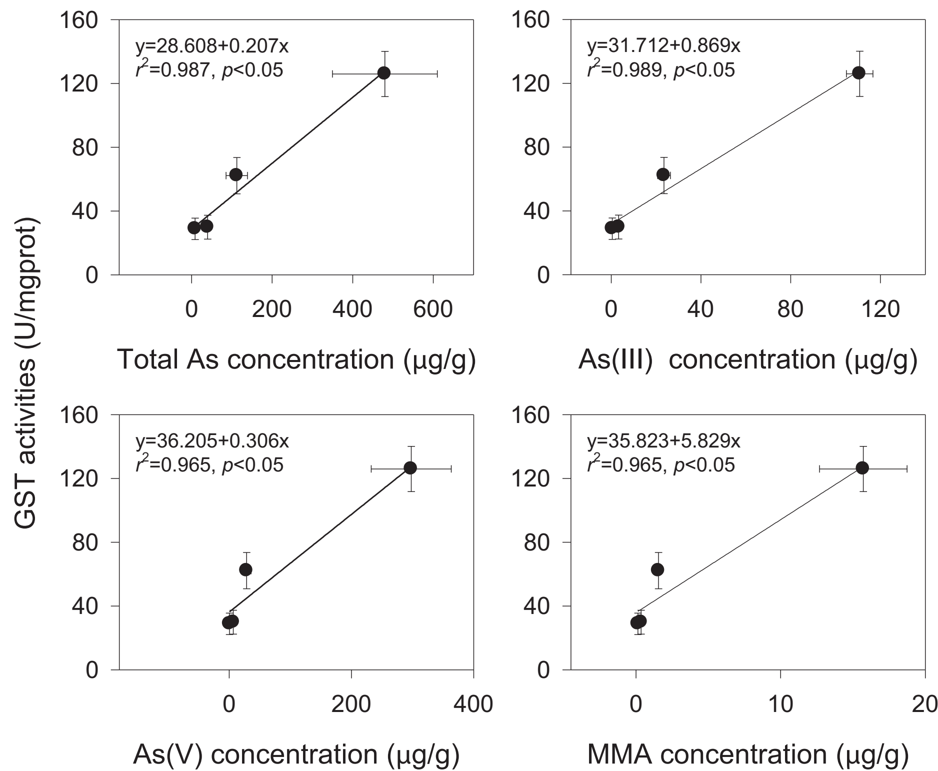

Fig. 4. The correlation between GST activities and total As, As(III), As(V) and MMA concentrations in the oysters after waterborne As(V) exposure for 30 days. As(III), arsenite; As(V), arsenate; MMA, monomethylarsonate. Data are mean ± SD (n = 6).

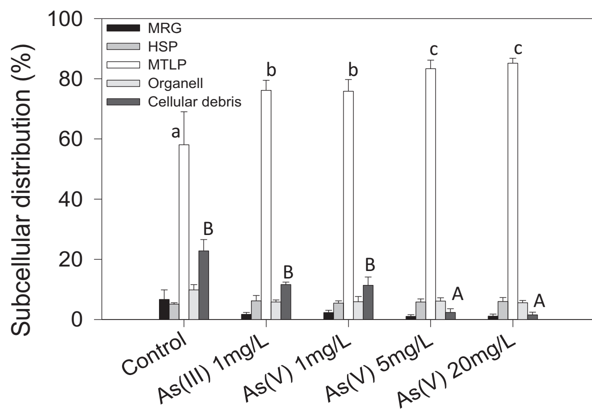

Fig. 5. Subcellular As distribution (%) in the oysters after waterborne inorganic As exposure for 30 days. Data are mean ± SD (n = 6). MRG, metal-rich granules; HSP, heat-sensitive protein; MTLP, metallothionein-like proteins. Different letters represent significant difference between treatments (p < 0.05).

4. Discussion

4.1. Arsenic bioaccumulation

Body As concentration increased significantly after all waterborne As exposure treatments except 1 mg/L As(V), and the As bioaccumulation in the oysters was proportional with the As(V) waterborne exposure concentrations, similar to the previous study in freshwater fish *Tilapia mossambica* (Suhendrayatna et al., 2002b) and *Oryzias latipes* (Suhendrayatna et al., 2002a). Suhendrayatna et al. (2002b) measured As bioaccumulation in *T. mossambica* following the exposure of 5, 10, 15 mg As(III)/L or 0.1, 5, 10 mg As(V)/L and they found the accumulation of As by *T. mossambica* was proportional to the concentration of As in water. Ünlü and Fowler (1979) found that the amount of As absorbed by purple mussel *Mytilus galloprovincialis* was not proportional to the seawater As concentrations. In contrast, Gailer et al. (1995) exposed mussels *Mytilus edulis* to 100 µg/L dissolved arsenite or arsenate for 10 days and reported that the mussels contained 5.8 and 3.9 µg/kg wet wt total As, respectively, compared to 4.9 µg/g in control mussels, indicating that both inorganic As species have very low bioavailability (estimated at ~2% of bioavailability of arsenobetaine) and that As(III) was only slightly more bioavailable than As(V) in this mollusk (Gailer et al., 1995). And the bioaccumulation of As was very low and not proportional to the inorganic As exposure concentration in a marine juvenile grunt *Terapon jarbua* following waterborne 100 µg/L As(III) and As(V) for 10 days (Zhang et al., 2012). The present results demonstrated that the total As was not elevated in the oysters exposed to a higher level of As(V) (1 mg/L), again suggesting the low bioavailability of As(V) to the mollusks. In contrast, the As accumulation in the oysters was higher when exposed to As(III) than to As(V), demonstrating As(III) was more permeable to the epithelia or more easily metabolized by oyster compared to As(V) when facing high As exposure concentrations.

4.2. Biotransformation of arsenic speciation

It has been well known that As(V) reduction (from As(V) to As(III)) and subsequent methylation (from inorganic As to MMA and DMA) are the two steps of biotransformation in some aquatic organisms (reviewed by Vahter, 2002). The present study provided some new insights about the As biotransformation in oysters.

As(V) reduction to As(III) was certainly occurred in oysters in this study. In all the three As(V) exposure treatments, both the concentration and proportion of tissue As(III) increased significantly. Many researchers have considered As(V) reduction as a detoxification process by nonenzymatically or enzymatically reacting with GSH, e.g. in carps (Shiomi et al., 1996), lichen *Hypogymnia physodes*

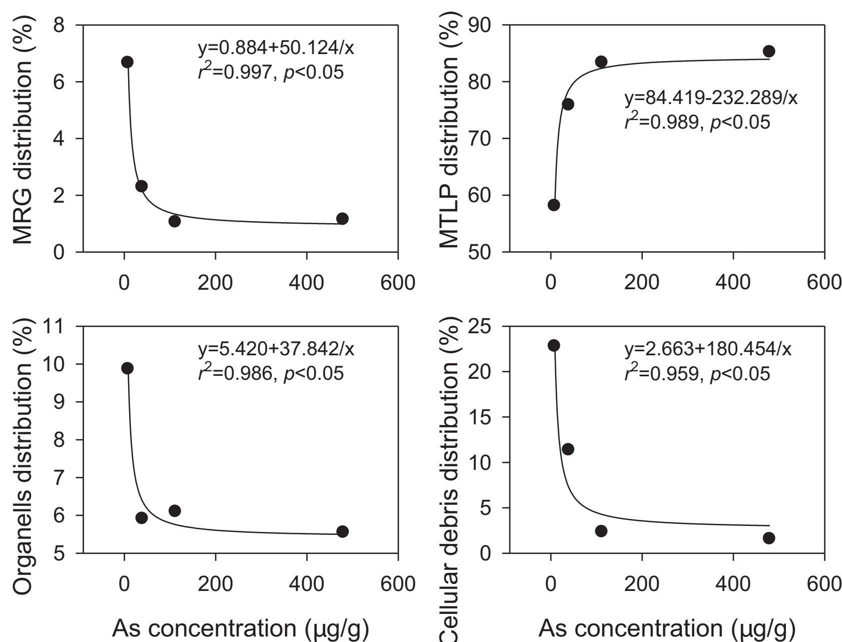

Fig. 6. The correlation between total As and MRG, MTLP, organelles, and cellular debris in the oysters after waterborne As(V) exposure for 30 days. MRG, metal-rich granules; MTLP, metallothionein-like proteins.

(Mrak et al., 2008), and oligochaetes (Erickson et al., 2011). While, we also observed As(III) could be oxidized to As(V) in oysters. In the As(III) exposed oysters, the concentration of tissue As(V) had a 20× increase, although its proportion was comparable to the control. Similar results were rare to be reported but in some microalgae. In a yellowstone thermoacidophilic eukaryotic alga *Cyanidioschyzon* sp., both the oxidation and reduction between As(III) and As(V) were found in parallel (Qin et al., 2009). As(III) oxidation was also observed with a higher intracellular proportion of As(V) in the freshwater green alga *Chlamydomonas reinhardtii* under As(III) exposure or the nutrient-limited conditions (Wang et al., 2013). The mechanism of As(III) oxidation in organisms has not be clear yet. It should be noted that in control oysters, As(III) and As(V) presented in a closely 1:1 ratio (Table 1), suggesting it was the balance status between As(III) and As(V) at the natural oyster tissue redox potential. Therefore, it was make sense that when exceeded As(III) entered the body, the chemical equilibrium between As(III) and As(V) was broken, and more As(V) could be transformed from As(III). In this way, the tissue redox potential may become the key factor to control the ratio of inorganic As(III) and As(V) in organisms, which will be worth to investigate further in the future.

In this study, inorganic As was the only source for oysters uptake from the environment, however, inorganic As was only a small fraction of the total As accumulation (10.1%) in control oysters. Similar results were found that inorganic As consisted 1.4%, 10.0%, 3.0%, 1.6%, and 1.3% of the total As in oysters *C. gigas* from Taiwan, Japan, and northwest of Spain (Edmonds and Francesconi, 1993; Han et al., 1998; Kohlmeyer et al., 2002; Liu et al., 2006, 2008). These results indicated inorganic As could be biotransformed efficiently to the organic forms in oysters in the natural environment. Since organic As was considered as less toxic forms than inorganic forms, it also indicated oyster could detoxify As in such conditions. In contrast, this study also found inorganic As consisted significantly more of the total As in the high As exposures (especially 5 and 20 mg/L As(V)), demonstrating As uptake exceeded the maximum inorganic As biotransformation rate plus excretion, resulting in the incomplete biotransformation. And the differences in inorganic As biotransformation to organic As compounds with increasing concentrations of dissolved As(III) and As(V) can be explained in part as resulting from different As biotransformation kinetics at different high As exposure concentrations. However, As biotransformation kinetics at different high As exposure concentrations in oysters needs further investigation.

Methylation of As(III) to MMA and DMA has been well known as the critical process of As biotransformation from the inorganic species to the organic species. The incomplete biotransformation of inorganic As species in high As exposed oysters was thus possibly due to the saturation of the methylation capacity. Yin et al. (2011) also reported that As(III) methylation could be saturated in a model protozoan *Tetrahymena thermophila* at external 40 μM As(V) exposure. MMA levels were the lowest in all treatments, suggesting that the inorganic As was methylated at limited rates but MMA was metabolized or biotransformed to the other species (e.g. DMA) quickly. Cullen et al. (1994) also found MMA was metabolized faster than As(V), As(III), and DMA in two microorganisms *Apiotrichum humicola* and *Scopulariopsis brevicaulis*. Compared to MMA, DMA consisted more proportions of the total As, suggesting DMA was a more stable form in oysters.

After methylation, different organisms have different strategies for As detoxification. This study found that oysters were apt to subsequently biotransform MMA and DMA to AsB, one of the least toxic and reactive As species. AsB was predominant in control and low As exposed oysters, which was similar to many earlier studies. Vilanó and Rubio (2001) showed that three As species were detected in oyster tissues, AsB (87%), a probable AsS (4.9%), and DMA (4.7%). Li et al. (2003) reported that AsS (14.4–55.2%) along with AsB (37.3–82.3%) were the major As species in oyster and clam. Bergés-Tiznado et al. (2013) found that AsB was the major arseno-compound (43.2–76.3%) in the cultured oyster *Crassostrea gigas* and *Crassostrea corteziensis*. Our previous study also found that AsB constituted 83.0–95.1% as the predominant As species in oyster *S. cucullata* in the intertidal zone (Zhang et al., 2013). In total, these results demonstrated that AsB was probably an end product of As biotransformation, and AsB was apt to be accumulated in the body rather than excreted in oysters.

It was also interesting that AsB might be elevated in different strategies by As (III) and As(V) exposure. AsB levels were significantly lower in As(V) exposure than As(III) exposures. Even

in 20 mg/L As(V) exposed oysters, the inorganic and methylated As levels were more than 10× higher, AsB levels was just about 1/3 comparing to the ones in As(III) exposed oysters. This results demonstrated that long-term As(III) exposure might induce some AsB synthesis metabolisms that As(V) exposure cannot, which could change the different inorganic As biotransformation kinetics. Although the AsB synthesis pathways are still unclear, it was no doubt that the biotransformation to AsB was saturated when the tissue inorganic As was exceeded, since both the concentration and proportion of AsB were significantly reduced in 5 mg/L and 20 mg/L As(V) exposed oysters. In parallel, the motilities in these two treatments were also increased, demonstrating As toxicity occurred when toxic As species could not be totally converted to AsB. This phenomenon indicated that the proportion of AsB could become a good indicator for As toxicity or stress in oysters.

4.3. Glutathione-S-transferases activities

In this study, GST activities were positive correlated with the total As, As(III), As(V), and MMA concentrations, suggesting that GST might play an important role in multiple steps of As biotransformation and detoxification process, probably a biotransformation rate limiting enzyme in oysters. And the massive bioaccumulation of dissolved As(III) and As(V) in oysters probably led to binding of As to GSH. Besides functioning in inorganic As(V) reduction as described in Section 4.2, GST was also considered to act in MMA(V) and DMA(V) reduction (to MMA(III) and DMA(III), respectively) in human (Aposhian et al., 2004). Aposhian et al. (2004) also suggested GST activity represents the rate limiting process for biotransformation of inorganic As. Bagnyukova et al. (2007) reported that GSH was increased in liver of fish exposed to inorganic As, and pointed GSH as a common and important mechanism of protection during As detoxification and metabolic processes. It should be noted that GST activity was elevated significantly in 1 mg/L As(III) exposed oysters than 1 mg/L As(V) exposed ones, although the latter required more GST to confront more oxidative stress theoretically. It suggested GST might also played other roles than As(V) reduction to reduce As toxicity, e.g. for As(III) GSH binding to protect thiol-containing proteins. Overall, the results of this study proposed GST as a potential biomarker of As pollution in oysters due to the good relationship between GST and As bioaccumulation. More studies are required to characterize the role of GST in As biotransformation and detoxification in oysters.

4.4. Subcellular distribution

After 30 days exposure, MTLP was the major binding site for As in the oyster tissues, followed by cellular debris. By comparison, only a small fraction of As was found in MRG, organelles, and HSP after waterborne exposure. These data were consistent with our previous study that MTLP was the major binding site for As in clams and fish, and cellular debris was another important fraction after dietborne As exposure. In contrast, only a small fraction of As was bound with MRG, organelles, and HSP (Zhang et al., 2011, 2012). Yu et al. (2013) also reported that MTLP was often the dominant fraction, cellular debris and organelles were important in binding As, while MRG was only a small fraction in the oyster *C. hongkongensis*. And subcellular As distribution depended on the As concentration in the oyster tissues, which was accordance with this study. In this study, As bioaccumulation in exposed oysters affected the distribution of subcellular level. More As was bioaccumulated in exposed oysters, and more As was distributed in the MTLP (detoxification pool). More As distributed in the subcellular pools such as MTLP fraction in the As exposed oysters, suggesting there was another detoxification strategy. Since a large proportion of As (mainly AsB) was distributed in the MTLP fraction, As might have been detoxified. MTLP was generally taken as a detoxification mechanism by organisms due to its latent capacity to bind metals for cysteine contents. Recently, several studies on aquatic organisms have highlighted the significance of MTLP in metal detoxification. Chowdhury et al. (2005) reported the dominance of As in MTLP fraction, indicating that metallothioneins might protect against As toxicity at the cellular levels. In earlier study, As contamination induced metallothioneins in liver particularly in freshwater teleosts *Channa punctatus* (Roy and Bhattacharya, 2006).

5. Conclusion

In summary, the total As and given inorganic and organic As species in oysters was elevated by very high concentrations of waterborne inorganic As exposure. GST, a possible rate limiting enzyme of As biotransformation, was also elevated. The efficiency of these As biotransformation was decreased when the load of As was exceeded. The inorganic As species were biotransformated into methylarsenic (MMA and DMA) and less toxic AsB, and sequestered in the detoxified MTLP fractions. It demonstrated at least two types of As detoxification strategies (biotransformation and binding with MTLP) existed in the oysters. It was obvious that As biotransformation was different between As(III) and As(V) exposed oysters. Therefore, the further mechanisms of As biotransformation and detoxification in oysters are necessary to be investigated in the future studies.

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