A metabolomic investigation on arsenic-induced toxicological effects in the clam *Ruditapes philippinarum* under different salinities

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ABSTRACT

Arsenic is an important contaminant in the Bohai marine ecosystem due to the anthropogenic activities. In this work, we investigated the toxicological effects of arsenic in *Ruditapes philippinarum* under different seawater salinities using NMR-based metabolomics. Under normal salinity (31.1 ppt), arsenic decreased the levels of amino acids (glutamate, β-alanine, etc.), and increased the levels of betaine and fumarate. The metabolic biomarkers including decreased threonine, histidine, ATP and fumarate were found in the muscles of arsenic-treated clams under medium salinity (23.3 ppt). However, only elevated ATP and depleted succinate were detected in the arsenic-exposed clam samples under low salinity (15.6 ppt). These differential metabolic biomarkers indicated that arsenic could induce osmotic stress and disturbance in energy metabolism in clam under normal and medium salinities. However, arsenic caused only disturbance in energy metabolism in clam under low salinity. Overall, our results demonstrated that seawater salinity could influence the toxicological effects of arsenic.

1. Introduction

Arsenic (As) is a known toxicant to organisms including both animals and plants (Baig and Kazi, 2012; Jain et al., 2011; Srivastava and D'Souza, 2010; Rana et al., 2010). It can easily enter into human body through food chains and has been recognized as a potent human carcinogen of skin, lung, bladder and kidney (Ventura-Lima et al., 2011). Due to the anthropogenic activities, arsenic can be released to the aquatic environment from the discharge of waste waters of mining, smelting, fertilizer and pesticides. In aquatic environment, arsenic can be found in many chemical forms including inorganic arsenate (As(V)) or arsenite (As(III)), organoarsenics such as arsenoribosides, arseno-betaine or monomethylarsonic acid (Harrington et al., 1997; Sohrin et al., 1997). However, the dominant form of arsenic is arsenate (As(V)) in waters (Maeda 1994; Suhendrayatna et al., 2002). In the certain polluted estuaries along the Bohai coast, the contamination of arsenic has been of great concern with a total arsenic concentration up to $400\,\mu g\,L^{-1}$ (Meng et al., 2004). Marine organisms can accumulate high amounts of arsenic from the aquatic environment. Ventura-Lima et al. (2011) has recently reviewed the toxicity of arsenic at biochemical levels in aquatic animals. Basically, arsenic can induce cytotoxicity and oxidative stress in both fishes and polychaete (Ventura-Lima et al., 2007, 2009; Bhattacharya and Bhattacharya, 2007).

Along the Bohai coast, Manila clam *Ruditapes philippinarum* thrives in both low intertidal and subtidal zones with changeable environmental salinities due to the entry of terrigenous fresh water. In marine ecotoxicology, *R. philippinarum* has been frequently used as a preferable bioindicator because of its wide distribution, long life history and high tolerance to environmental changes (e.g., salinity, temperature) (Ji et al., 2006; Laing and Child, 1996; Matozzo et al., 2004; Hegaret et al., 2007; Moraga et al., 2002; Zhang et al., 2011a,b; Liu et al., 2011a,b,c). Seawater salinity is the key environmental factor for *R. philippinarum*. However, the research on the influence of seawater salinity on the toxicological effects of arsenic, to our knowledge, is limited. In order to better the understanding of the toxicological mechanisms of contaminants, *R. philippinarum* was selected as the experimental animal in this work to elucidate the toxicological effects of arsenic under different salinities.

Metabolomics is a well-established technique in system biology and has been widely applied in toxicology (Bundy et al., 2004; Wu et al., 2005; Viant et al., 2006a,b; Jones et al., 2008; Wu and Wang, 2010, 2011). Basically, metabolomics focuses on the analysis of all low molecular weight ($< 1000\,Da$) metabolites

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that are the end products of metabolism, representing the physiological status of a biological system (cell, tissue, urine, etc.) at molecular level (Lindon et al., 2000). Among the most frequently applicable analytical techniques in metabolomics, including mass spectrometry (MS), high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and nuclear magnetic resonance (NMR) spectroscopy, NMR spectroscopy is suitable to measure a wide range of detectable endogenous metabolites simultaneously in a non-invasive and rapid manner (Viant et al., 2001; Brindle et al., 2002; Lindon et al., 2000). Combined with computer-based pattern recognition methods, such as principal components analysis (PCA), partial least square-discriminant analysis (PLS-DA) and genetic algorithms (GALGO), metabolomics can discover metabolic biomarkers that can provide an insight on the biochemical perturbations induced by either endogenous or exogenous factors (Lindon et al., 2000; Wang et al., 2003). To date, NMR-based metabolomics has been also efficiently used in marine biology and ecotoxicology (Viant et al., 2006a,b; Jones et al., 2008; Wu and Wang, 2010, 2011; Gordon and Leggat, 2010; Lannig et al., 2010; Tikunov et al., 2010).

The changeable environmental factors, such as seawater salinity and temperature, can affect the physiological status of animals, which could potentially influence the toxicological effects of contaminants in experimental animals (Hines et al., 2007a, 2007b; Morrison et al., 2007; Viant, 2006; Laing and Child, 1996). The aim of this work was designed to investigate the influences of different seawater salinities (31.1, 23.3 and 15.6 ppt) on the toxicological effects of arsenic in R. philippinarum using NMR-based metabolomics. The experimental salinities were ranged in the suitable salinities between 14.0 ppt and 33.5 ppt for clam. To unravel the influence of salinity on the toxicological effects of arsenic, the metabolic differences induced by arsenic in the adductor muscle of R. philippinarum under different salinities were characterized by NMR-based metabolomics.

2. Materials and methods

2.1. Chemicals

Sodium dihydrogen phosphate (Na_2HPO_4), disodium hydrogen phosphate (NaH_2PO_4) and sodium arsenate (Na_2HAsO_4) (all in analytical grade), and extraction solvents, methanol and chloroform (HPLC grade) were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China). Deuterium oxide (D_2O, 99.9% in D) and sodium 3-trimethlysilyl [2,2,3,3-D4] propionate (TSP) were purchased from Cambridge Isotope Laboratories (Miami, FL).

2.2. Clam exposure

Adult clams R. philippinarum (shell length: 3.4–3.8 cm, from White pedigree, $n=72$) were purchased from a local culturing farm. After transport to the laboratory, the animals were kept in natural seawater (31.1 ppt) collected from pristine environment for 1 d. Then, the clams were acclimatized at normal (31.1 ppt), 75% (23.3 ppt) and 50% (15.6 ppt) salinities of seawater (prepared from different ratios of seawater and de-ionized water). The salinities were reduced gradually over a period of 3 d to achieve the experimental salinities which were accurately measured daily. Clams were then acclimatized at each specific salinity for another 3 days, with water changed daily. Six treatments were designed including three salinity control groups (normal, 75% and 50% salinities of seawater) and three arsenic exposure groups under corresponding salinities. The nominal concentration of arsenic was 20 µg L^{-1} (prepared from Na_2HAsO_4) that is environmentally realistic in the polluted sites along the Bohai coast (Meng et al., 2004). In each treatment, twelve clams were maintained in a tank containing 10 L seawater with different salinities. During the acclimation and exposure periods, clams were kept at 22 °C under a photoperiod of 12 h light and 12 h dark, and fed with the Chlorella vulgaris Beij at a ratio of 2% tissue dry weight daily. After 48 h of exposure, all the clams were immediately dissected for adductor muscle tissues which were flash frozen in liquid nitrogen, and then stored at −80 °C before metabolite extraction ($n=12$).

2.3. Metabolite extraction

Polar metabolites in the adductor muscle tissues of clams were extracted by the modified extraction protocol as described previously (Zhang et al., 2011a; Liu et al., 2011a). Briefly, the adductor muscle tissue (ca. 50 mg) was homogenized and extracted in 4 mL g^{-1} of methanol, 5.25 mL g^{-1} of water and 2 mL g^{-1} of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts of muscle tissue were subsequently re-suspended in 600 µL phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4, including 0.5 mM TSP, pH 7.0) in D_2O. The mixture was vortexed and then centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 µL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.4. High resolution one dimensional ^1H NMR spectroscopy

Extracts of adductor muscles from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K) as described previously (Zhang et al., 2011b; Liu et al., 2011b,c). Briefly, one-dimensional ^1H NMR spectra were obtained using a 11.9 µs pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse sequence to suppress water peak, with 128 transients collected into 16,384 data points. Datasets were zero-filled to 32,768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All ^1H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker). NMR spectral peaks were assigned following the tabulated chemical shifts (Viant et al., 2003; Fan, 1996) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada).

2.5. Spectral pre-processing and multivariate data analysis

One dimensional proton NMR spectra were converted to a format for multivariate analysis using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA) (Purohit et al., 2004). Each spectrum was segmented into 0.005 ppm bins between 0.2 and 10.0 ppm with bins from 4.70 to 5.20 ppm (water) excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (g log) with transformation parameter $\lambda = 2.0 \times 10^{-9}$ (Purohit et al., 2004; Parsons et al., 2007) to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks. Data were mean-centered before principal components analysis (PCA) using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA).

As an unsupervised pattern recognition method, PCA was used to reduce the dimensionality of the data and summarize the similarities and differences between multiple NMR spectra (Xu, 2004). The algorithm of this pattern recognition method calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of variance. For each model built, the loading vector for the PC could be examined to identify the metabolites which contributed to the clusters. One-way analysis of variance (ANOVA) was conducted on the PC scores from each group to test the statistical significance ($p < 0.05$) of separations. The Chenomx software (Evaluation version, Chenomx Inc., Canada) was then used to identify and quantify the potentially significant metabolites between various groups (Hines et al., 2007a, 2007b). The metabolite concentrations were normalized to the mass of muscle tissue by calculating the concentrations of metabolites in each NMR tube.
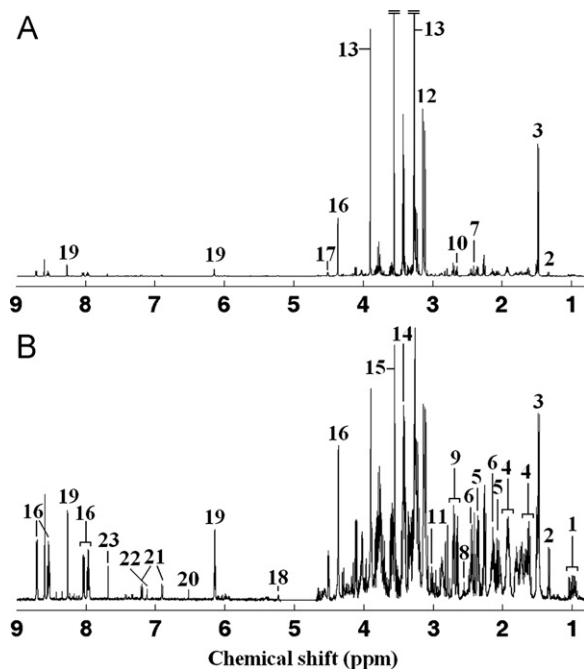
2.6. Statistical analysis

Metabolite concentrations were tested for normal distribution (Ryan–Joiner's test) and homogeneity of variances (Bartlett's test). Two-way ANOVA was used to compare the metabolite concentrations by a post-hoc test for pairwise comparisons (e.g., for comparison between two arsenic-blank and arsenic-treated groups under each salinity, or vice versa) (Munari et al., 2011). All metabolite concentrations are expressed as means ± standard deviation. Furthermore, one-way ANOVA combined with Tukey's test was conducted on the concentrations of each metabolite between the 'control' (arsenic-blank) and arsenic-exposed groups at each salinity, respectively. A p value less than 0.05 was considered statistically significant. Correlation analysis between the metabolite concentrations was carried out and R-square values are reported. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.
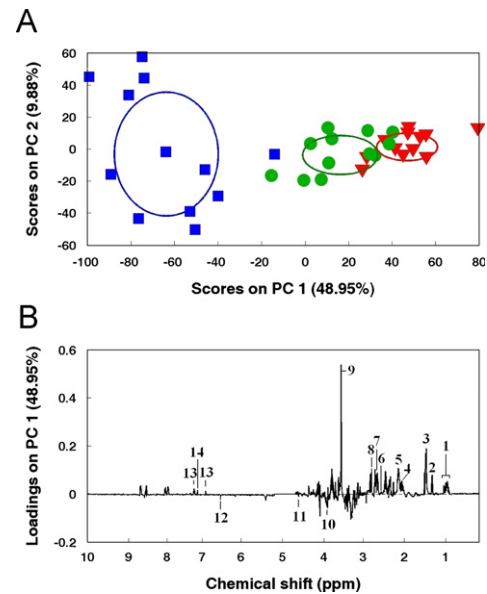
3. Results and discussion

As reported previously, the White pedigree of Manila clam R. philippinarum is relatively sensitive to heavy metal contaminants including mercury, cadmium and zinc (Liu et al., 2011b,c; Wu

et al., 2011). It was therefore selected as the experimental animal in this study. A representative ^{1}N NMR spectrum of muscle extracts was shown in Fig. 1 in both original and generalized log-transformed forms. Several classes of metabolites were identified by Chenomx software and labeled in Fig. 1, including amino acids (valine, leucine, isoleucine, alanine, threonine, arginine, glutamate, glutamine, β-alanine, aspartate, lysine, glycine, tyrosine and histidine), organic osmolytes (hypotaurine, taurine, homarine and betaine), organic acids (succinate and fumarate), phosphagen (phosphocholine) and energy storage compounds (ATP and glucose).

Principal components analysis (PCA) was performed on the NMR spectral datasets of adductor muscle extracts from normal seawater 'control' (31.1 ppt, inverted red triangles) and normal seawater with reduced salinity 'control' clam groups (23.3 ppt, green cycles; 15.6 ppt, blue squares) to compare the metabolic profiles. The significant separations ($p < 0.001$) were found between the groups using PCA (Fig. 2A), which meant that there were significant metabolic differences between these three groups of clam samples under different salinities. The potentially significant metabolic responses were labeled in the loadings plot (Fig. 2). Hereby, one question has arisen: what differential toxicological effects could be induced by arsenic in *R. philippinarum* under hypoosmotic status caused by reduced salinity? To compare the metabolic differences induced by arsenic under different salinities, PCA was performed on the ^{1}H NMR spectral data sets generated from the 'control' (arsenic-blank) and arsenic-treated groups of clams under corresponding reduced seawater salinities, respectively (Fig. 2). For the groups under normal (31.1 ppt) and reduced seawater salinities (23.3 and 15.6 ppt), the 'control' (arsenic-blank, inverted red triangles) and arsenic-exposed groups (green cycles) were all significantly separated
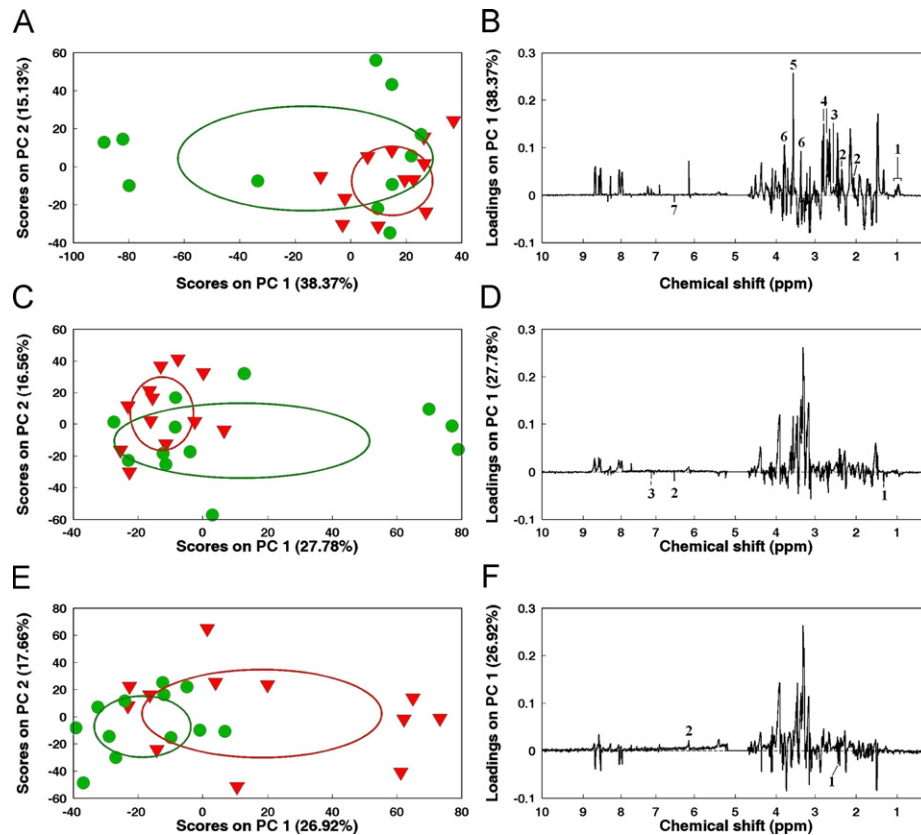
Fig. 2. Principal components analysis (PCA) on the ^{1}H NMR spectra from clam muscles showing significant separations between normal seawater salinity (▼) and reduced salinity-treated (75% normal seawater salinity, 23.3 ppt, ●; 50% normal seawater salinity, 15.6 ppt, ■) groups (A) and corresponding PC1 loadings plots (B) showing the metabolic differences between the groups. Ellipses represented mean ± standard deviation of PC scores for each group. Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) threonine, (3) alanine, (4) glutamate, (5) glutamine, (6) β-alanine, (7) hypotaurine, (8) aspartate, (9) glycine, (10) betaine, (11) β-glucose, (12) fumarate, (13) tyrosine and (14) histidine.

along PC1 axis with corresponding statistical significances of 0.042, 0.049 and 0.004 (Fig. 3A, C and E).

Two-way ANOVA analysis was then conducted on the metabolite concentrations revealing statistical significances of metabolites caused by salinity, arsenic and salinity/arsenic ($p < 0.05$) (Table 1). Obviously, the concentrations of amino acids including valine, leucine, isoleucine, threonine, alanine, glutamate, glutamine, β-alanine, aspartate, glycine, tyrosine and histidine were significantly ($p < 0.001$) decreased in adductor muscles of *R. philippinarum* under reduced salinities (Table 2). Two organic osmolytes, hypotaurine and betaine, were significantly ($p < 0.001$) elevated and depleted, respectively. Reduced salinity can induce hypoosmotic stress to marine mollusks. As reported by Jones et al. (2008) and Viant et al. (2003), some marine mollusks use high intracellular concentrations of free amino acids to balance their intracellular osmolarity with the environment (Jones et al., 2008; Viant et al., 2003). In our case, the concentrations of amino acids were reduced to balance the osmolarity under reduced seawater salinities together with the reduced osmolyte, hypotaurine. Correlation analysis indicated positive coefficients ($R^2 > 0.25$) between hypotaurine and amino acids including valine, isoleucine, leucine, threonine, alanine, glutamine, β-alanine, tyrosine and histidine, with p values less than 0.01. It confirmed that these amino acids were involved in osmotic regulation in clam. Interestingly, the osmolyte, betaine was increased, which was different to the alteration of hypotaurine. In diseased abalone, decreased concentrations of amino acid were negatively correlated with another osmolyte, homarine that was increased (Viant et al., 2003). In clam *R. philippinarum* under reduced salinities, the increased betaine could be related to the compensatory mechanism to help balance the loss of other osmolytes, such as amino acids and hypotaurine, which was demonstrated by the negative correlation coefficients between betaine and hypotaurine, as well as between betaine and amino

Fig. 1. A representative 1-dimensional 500 MHz ^{1}H NMR spectrum of adductor muscle tissue extracts from a white clam of control group under normal seawater salinity (31.1 ppt) in original (A) and generalized log transformed ($\lambda = 2.0 \times 10^{-9}$) (B) forms. Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) threonine, (3) alanine, (4) arginine, (5) glutamate, (6) glutamine, (7) succinate, (8) β-alanine, (9) aspartate, (10) hypotaurine, (11) lysine, (12) phosphocholine, (13) betaine, (14) taurine, (15) glycine, (16) homarine, (17) β-glucose, (18) α-glucose, (19) ATP, (20) fumarate, (21) tyrosine, (22) histidine and (23) unknown 1 (7.68 ppm).

Fig. 3. Principal components analysis (PCA) on the ^{1}H NMR spectra of both arsenic-blank (▼) and arsenic-exposed (●) clam samples under reduced salinities (normal seawater salinity, A; 75% normal seawater salinity, 23.3 ppt, C; 50% normal seawater salinity, 15.6 ppt, (E), and corresponding PC1 loadings plots, (B), (D) and (F) showing the metabolic differences between the arsenic-blank and arsenic-exposed clam samples after 48 h of exposure. Ellipses represented mean ± SD of PC scores for each group. Keys in (B): (1) branched chain amino acids: isoleucine and leucine, (2) glutamate, (3) β-alanine, (4) aspartate, (5) glycine, (6) betaine and (7) fumarate; keys in (D): (1) threonine, (2) fumarate and (3) histidine; keys in (F): (1) succinate and (2) ATP.

acids ($p < 0.01$) mentioned above (Viant et al., 2003). In addition, glucose was significantly ($p < 0.05$) decreased, whilst fumarate was significantly ($p < 0.05$) increased. Glucose is an energy storage compound and fumarate is an intermediate in the citric acid cycle used by cells to produce energy in the form of ATP from food. Since hypoosmotic stress can significantly reduce the food intake in clams, the experimental clams under low salinities were believed under restriction of food during acclimation and exposure. Therefore, the depleted glucose and elevated fumarate were linked to the metabolic disturbance in energy metabolism caused by reduced salinity-induced starvation.

From the analysis of two-way ANOVA, arsenic induced significant metabolic changes including leucine, alanine, glutamate, succinate, β-alanine, betaine and ATP, together with some other metabolites, such as glycine and tyrosine, with statistical significances approaching 0.05. To further the comparison on the metabolic profiles, one-way ANOVA analysis was conducted on the metabolite concentrations from both arsenic-treated and arsenic-blank clam samples under certain salinity (Table 2). Clearly, arsenic exposure decreased the levels of amino acids (leucine, isoleucine, glutamate, β-alanine, aspartate and tyrosine) and increased the levels of betaine and fumarate in clam muscles under normal salinity (31.1 ppt). Similarly, these metabolic biomarkers indicated that arsenic induced osmotic stress and disturbance in energy metabolism compared to the metabolic changes induced by reduced salinities in *R. philippinarum*. The metabolic biomarkers from arsenic-exposed clam samples under reduced salinities were distinctively different. Under medium salinity (23.3 ppt), arsenic depleted the levels of threonine, histidine, ATP and fumarate in

clam muscles. These differential metabolic biomarkers demonstrated the osmotic stress and disturbance in energy metabolism induced by arsenic through different metabolic pathways in clam samples under medium salinity (23.3 ppt). However, only increased succinate and decreased ATP were found in the clam samples under low salinity (15.6 ppt). Similar to marine mussels that are facultative anaerobes, clam might potentially utilize a peculiar carbohydrate fermentation process, with a non-oxidative TCA cycle that works in reverse, from pyruvate up to succinate (Jones et al., 2008; De Zwaan et al., 1976). Therefore the increased succinate and decreased ATP indicated the disturbance in energy metabolism through carbohydrate fermentation process.

4. Conclusions

Environmental factors (e.g., temperature and salinity) can potentially influence the toxicological responses of marine environmental contaminants in experimental animals. In this study, the influences of different seawater salinities on the toxicological effects are investigated in the clam *R. philippinarum* exposed to environmentally relevant arsenic (20 μg L^{-1}) using NMR-based metabolomics. Under normal seawater salinity (31.1 ppt) and medium salinity (23.3 ppt), arsenic mainly induced both osmotic stress and disturbances in energy metabolism in adductor muscles of clams via different toxicological mechanisms based on the differential metabolic biomarkers. The elevated succinate and depleted ATP suggested the disturbance in energy metabolism induced by arsenic under the low salinity of 15.6 ppt. Overall, our

Table 1
Two-way analysis of variance (ANOVA) on the metabolite concentrations in clam muscle tissues. Statistically significant results are indicated in bold.

Metabolites	Chemical shift (ppm, multiplicity)	p values		
		Salinity	Arsenic	Salinity × Arsenic
Amino acids				
Valine	1.05 (d)	**< 0.001**	0.123	0.272
Isoleucine	1.00 (d)	**< 0.001**	0.195	0.442
Leucine	0.94 (t)	**< 0.001**	**0.023**	0.227
Threonine	1.34 (d)	**< 0.001**	0.133	0.689
Alanine	1.48 (d)	**< 0.001**	**0.034**	0.243
Arginine	1.70 (m)	0.313	0.223	0.109
Glutamate	2.05 (m)	**< 0.001**	**0.043**	0.838
Glutamine	2.14 (m)	**< 0.001**	0.288	0.346
β-alanine	2.56 (t)	**< 0.001**	**0.023**	**0.040**
Aspartate	2.68 (ABX)	**< 0.001**	0.439	0.105
Lysine	3.03 (t)	0.120	0.097	0.565
Glycine	3.56 (s)	**< 0.001**	0.074	0.245
Tyrosine	6.91 (d)	**< 0.001**	0.062	**0.027**
Histidine	7.12 (d)	**< 0.001**	0.058	0.101
Organic osmolytes				
Hypotaurine	2.66 (t)	**< 0.001**	0.755	0.727
Taurine	3.27 (t)	0.672	0.874	0.799
Betaine	3.91 (s)	**< 0.001**	**0.009**	0.872
Homarine	4.37 (s)	0.130	0.672	0.406
Phosphagen				
Phosphocholine	3.22 (s)	0.399	0.105	0.081
Nucleotide				
ATP	6.15 (d)	0.127	**0.001**	0.225
Organic acids				
Succinate	2.41 (s)	0.260	**0.033**	0.585
Fumarate	6.52 (s)	**0.008**	0.660	**0.011**
Carbonhydrate				
Glucose	4.64 (d), 5.24 (d)	**0.011**	0.495	0.632
Miscellaneous				
Acetoacetate	2.26 (s)	0.057	0.132	0.385

s=singlet, d=doublet, t=triplet, m=multiplet, ABX=complex multiplet involving 2 protons (A and B) and a heavy atom (X).

Table 2
Metabolite concentrations (µmol g^{-1} wet tissue) in muscle tissues from R. philippinarum exposed to various salinity and combined salinity and arsenic (20 µg L^{-1}). Values are presented as mean ± standard deviation.

Metabolite[a]	Exposures					
	31.1 ppt	23.3 ppt	15.6 ppt	31.1 ppt+As	23.3 ppt+As	15.6 ppt+As
Valine	0.131 ± 0.034	0.098 ± 0.017	0.035 ± 0.011	0.110 ± 0.011	0.077 ± 0.033	0.039 ± 0.014
Isoleucine	0.105 ± 0.023	0.078 ± 0.012	0.031 ± 0.011	**0.085 ± 0.012**[#]	0.072 ± 0.033	0.031 ± 0.012
Leucine	0.120 ± 0.025	0.093 ± 0.017	0.034 ± 0.011	**0.096 ± 0.020**[#]	0.071 ± 0.025	0.034 ± 0.014
Threonine	0.370 ± 0.042	0.318 ± 0.110	0.120 ± 0.060	0.336 ± 0.113	**0.240 ± 0.107**[#]	0.100 ± 0.040
Alanine	14.075 ± 4.165	7.670 ± 3.141	2.836 ± 1.319	9.988 ± 3.752	5.517 ± 2.171	2.751 ± 0.890
Glutamate	3.879 ± 0.286	4.010 ± 0.736	2.495 ± 0.600	**3.292 ± 0.667**[#]	3.516 ± 0.902	2.214 ± 0.500
Glutamine	2.151 ± 0.483	1.526 ± 0.522	0.790 ± 0.481	1.540 ± 0.826	1.679 ± 0.925	0.565 ± 0.387
β-alanine	0.150 ± 0.038	0.039 ± 0.018	0.025 ± 0.010	**0.101 ± 0.038**[*]	0.033 ± 0.009	0.023 ± 0.007
Aspartate	4.514 ± 0.865	2.874 ± 0.972	1.385 ± 0.870	**3.143 ± 1.482**[#]	3.639 ± 1.618	1.063 ± 1.079
Glycine	41.936 ± 7.402	20.208 ± 10.203	0.914 ± 1.140	**31.092 ± 12.328**[#]	17.013 ± 6.947	0.579 ± 0.323
Tyrosine	0.187 ± 0.027	0.098 ± 0.040	0.044 ± 0.012	**0.135 ± 0.025**[**]	0.094 ± 0.030	0.049 ± 0.017
Histidine	0.188 ± 0.027	0.155 ± 0.059	0.031 ± 0.032	0.161 ± 0.055	**0.089 ± 0.043**[*]	0.041 ± 0.023
Hypotaurine	1.581 ± 0.202	1.439 ± 0.455	0.804 ± 0.433	1.532 ± 0.641	1.672 ± 0.647	0.773 ± 0.369
Betaine	25.105 ± 1.118	30.232 ± 1.572	35.667 ± 2.854	**27.707 ± 2.315**[*]	32.268 ± 3.087	37.312 ± 1.869
ATP	2.194 ± 0.278	2.417 ± 0.255	2.641 ± 0.433	1.968 ± 0.386	**2.129 ± 0.212**[#]	**2.012 ± 0.137**[**]
Succinate	0.364 ± 0.127	0.404 ± 0.402	0.229 ± 0.093	0.433 ± 0.061	0.673 ± 0.368	**0.497 ± 0.339**[#]
Fumarate	0.039 ± 0.006	0.108 ± 0.044	0.076 ± 0.031	**0.063 ± 0.028**[#]	**0.064 ± 0.012**[*]	0.084 ± 0.023
Glucose	2.786 ± 0.756	2.694 ± 0.947	1.564 ± 0.928	2.881 ± 0.838	2.617 ± 0.761	2.101 ± 0.511

[a] Metabolites were significant determined by two-way ANOVA in either salinity- or combined salinity and arsenic-exposed R. philippinarum muscles.
[*] Statistical significances between combined salinity and arsenic-exposed and corresponding salinity-exposed R. philippinarum samples ($p < 0.05$) were determined by one-way ANOVA with Tukey's test.
[**] Statistical significances between combined salinity and arsenic-exposed and corresponding salinity-exposed R. philippinarum samples ($p < 0.01$) were determined by one-way ANOVA with Tukey's test.
[#] Meant that the statistical significance approached 0.05 ($p < 0.1$).

results clearly demonstrated that seawater salinity could influence the toxicological effects of marine environmental contaminants.

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