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## Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics

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### ABSTRACT

The Manila clam (*Ruditapes philippinarum*) has been considered a good sentinel species for metal pollution monitoring in estuarine tidal flats. Along the Bohai coast of China, there are dominantly distributed three species of clams (White, Liangdao Red and Zebra in Yantai population) endowed with distinct tolerances to environmental stressors. In this study, adductor muscle samples were collected from both control and acute mercury exposed White, Liangdao Red and Zebra clams, and the extracts were analyzed by NMR-based metabolomics to compare the metabolic profiles and responses to the acute mercury exposure to determine the most sensitive clam species capable of acting as abioindicator for heavy metal pollution monitoring. The major abundant metabolites in the White clam sample were branched-chain amino acids (leucine, isoleucine and valine), lactate, arginine, aspartate, acetylcholine, homarine and ATP/ADP, while the metabolite profile of Zebra clam sample comprised high levels of glutamine, acetoacetate, betaine, taurine and one unidentified metabolite. For the Liangdao Red clam sample, the metabolite profile relatively exhibited high amount of branched-chain amino acids, arginine, glutamate, succinate, acetylcholine, homarine and two unassigned metabolites. After 48 h exposure of  $20 \mu\text{g L}^{-1} \text{Hg}^{2+}$ , the metabolic profiles showed significant differences between three clam species, which included increased lactate, succinate, taurine, acetylcholine, betaine and homarine and decreased alanine, arginine, glutamine, glutamate, acetoacetate, glycine and ATP/ADP in White clam samples, and elevated succinate, taurine and acetylcholine, and declined glutamine, glycine, and aspartate in Liangdao Red clam samples, while the increased branched-chain amino acids, lactate, succinate, acetylcholine and homarine, and reduced alanine, acetoacetate, glycine and taurine were observed in the Zebra clam samples. Overall, our findings showed that White clams could be a preferable bioindicator for the metal pollution monitoring based on the more sensitive metabolic changes in the adductor muscle compared with other two (Liangdao Red and Zebra) clam species.

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## 1. Introduction

The Bohai marine and coastal environment has been heavily polluted by several heavy metals including mercury (Hg), cadmium (Cd), lead (Pb), zinc (Zn), etc. due to the industrial discharge of heavy metals (Zhang, 2001). It is therefore necessary to assess the ecological risk of heavy metals in the marine and coastal environments. Marine mussels and oysters have been often used as sentinel organisms in many countries for marine and coast pollution monitoring since the 'Mussel Watch Program' was proposed in the late 1970s (Goldberg, 1975). The Manila clam, *Ruditapes philippinarum*, is consumed as an economic seafood and distributed in the natural environment along the coasts of China, Japan and Korea. It has also been suggested as a good bioindicator for the metal pollution monitoring of marine and coast ecosystems due to its wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of heavy metals (Park et al., 2008, 2006; Ji et al., 2006). Thus, it has been a bioindicator in the 'Mussel Watch Program' launched in China and frequently applied in the marine and coast environmental toxicology (Xu et al., 2010; Liu et al., 2010; Ji et al., 2006). There are widely distributed several species of clams (White, Liangdao Red, and Zebra) along the east and Bohai coast of China, which possess distinguishable tolerance to environmental stressors (Zhang et al., 2008). However, there is a lack of toxicological indices such as uptake rates, bioaccumulation and efflux rates, biochemical indices, or molecular biomarkers upon the tolerance and sensitivity of different species of clams to the heavy metal stresses. Therefore, it is extremely important to define a sensitive species of clam as sentinel for the heavy metal monitoring of the Bohai marine and coastal ecosystems.

Mercury is a hazardous contaminant in the marine and coastal environments due to its high toxicity to living organisms and the subsequent ecological risk (Zhang, 2001; Beiras et al., 2002). The predominant form of mercury contamination is  $\text{Hg}^{2+}$  that is highly water-soluble and readily accumulates in marine invertebrates. Along the Bohai marine and coastal environments, mercury pollution has posed an extremely high risk to the ecosystems and human health because of the high mercury concentration of up to  $100 \mu\text{g L}^{-1}$  seawater in certain polluted coastal environment (Zhang, 2001).

Metabolomics is a recent developed 'omic' technique that focuses on the systematic study of metabolic fingerprints (metabolic biomarkers), especially the small-molecule metabolites, left behind by the cellular processes (Davis, 2005). Such study of metabolic profiling has been widely used in drug toxicity, inborn disease diagnosis, functional genomics, and environmental toxicology (Brindle et al., 2002; Bundy et al., 2004; Wu et al., 2005a; Viant et al., 2006a,b). Several analytical technologies are well established and frequently applied in metabolomic studies, including proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy (Nicholson et al., 1985; Wang et al., 2003), gas chromatography-mass spectrometry (GC-MS) (Plumb et al., 2003), and liquid chromatography-mass spectrometry (LC-MS) (Ippolito et al., 2005).  $^1\text{H}$  NMR spectroscopy is rapid and rich in structural and quantitative information and allows the endogenous metabolites to be analyzed simul-

taneously (Lindon et al., 2000). Therefore, this technique is uniquely applicable to measure a wide range of metabolites from organs, tissues or tissue extracts to provide valuable biological and biochemical information on the biochemical perturbations induced by both endogenous and exogenous factors (Lindon et al., 2000; Wu et al., 2005b). In this limited study, we applied  $^1\text{H}$  NMR-based metabolomics to Manila clams (*R. philippinarum*) to (1) compare the differences between the metabolic profiles of adductor muscle tissue extracts from three species (White, Liangdao Red and Zebra) of clams and (2) compare the metabolic responses (molecular biomarkers) to the acute waterborne  $\text{Hg}^{2+}$  exposure to determine a sensitive species of clam as sentinel organism for the metal pollution monitoring of Bohai marine and coastal environments.

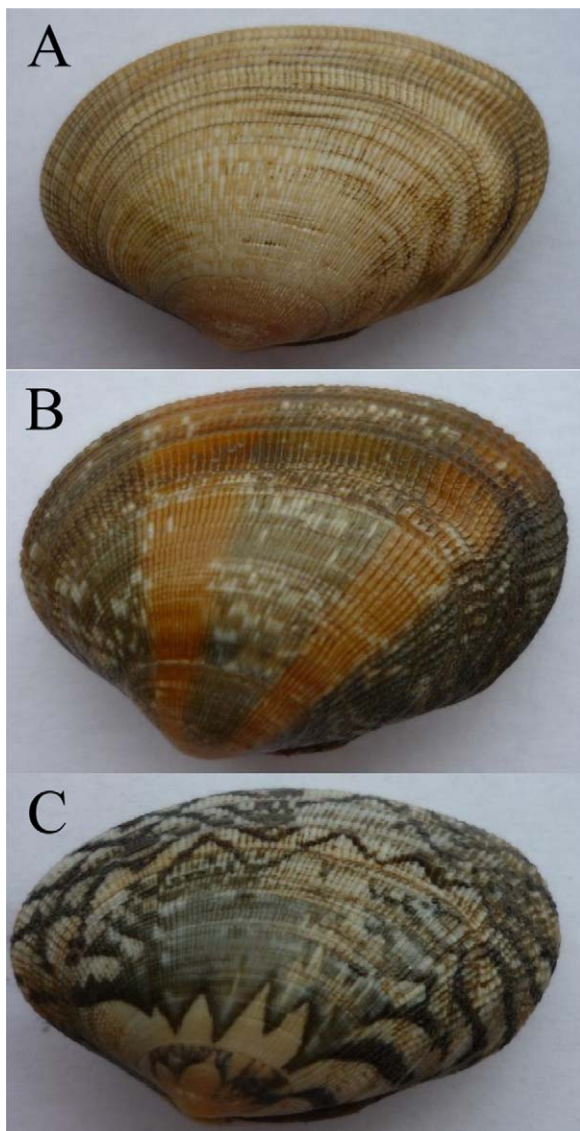
## 2. Materials and methods

### 2.1. Experimental design

All the adult Manila clams *R. philippinarum* (shell length: 3.4–3.8 cm, from White, Liangdao Red and Zebra species, Fig. 1) were purchased from a local unpolluted culturing farm. They were allowed to acclimatize in aerated seawater ( $25^\circ\text{C}$ , 33 psu, collected from pristine environment) in the laboratory and fed with *Chlorella vulgaris* Beij at a ration of 2% tissue dry weight per day. After acclimatization for 1 week, fifteen clams ( $n=5$  from White, Liangdao Red and Zebra species, respectively) were sacrificed and the adductor muscle tissues dissected from each individual. The remaining 30 clams ( $n=10$  from White, Liangdao Red and Zebra species) were divided into two tanks (one control and one mercury exposed) containing 5 White, Liangdao Red and Zebra clams, respectively, were exposed to dissolved  $20 \mu\text{g L}^{-1}$   $\text{Hg}^{2+}$  for 48 h. Mercury was prepared from  $\text{HgCl}_2$  (analytical grades). The experimental concentration of  $\text{Hg}^{2+}$  can be found in heavily polluted sites of Bohai Sea in which the mercury concentrations are ranged from 0.2 to  $166 \mu\text{g L}^{-1}$  seawater (Zhang, 2001). After 48 h of exposure, all the individual clams from both control and exposed groups were immediately dissected for the adductor muscle tissues. All the adductor muscle tissues were first frozen in liquid nitrogen, and then stored at  $-80^\circ\text{C}$  prior to the NMR analysis.

### 2.2. Metabolite extraction

Polar metabolites were extracted from adductor muscle tissues of clams by a modified extraction protocol using methanol/chloroform (Bligh and Dyer, 1959; Lin et al., 2007; Wu et al., 2008). Briefly, the adductor muscle tissue (ca. 100 mg) was homogenized and extracted in  $4 \text{ mL g}^{-1}$  of methanol,  $0.85 \text{ mL g}^{-1}$  of water, and  $2 \text{ mL g}^{-1}$  of chloroform. The mixture was shaken and centrifuged (5 min,  $3000 \times g$ , at  $4^\circ\text{C}$ ), and the supernatant substance was removed. A total of  $2 \text{ mL g}^{-1}$  of chloroform and  $2 \text{ mL g}^{-1}$  of water was added to the supernatant, and the mixture was vortexed and then centrifuged again (10 min,  $3000 \times g$ ,  $4^\circ\text{C}$ ). The methanol/water layer with polar metabolites was transferred to a glass vial. The sample was dried in a centrifugal concentrator and stored at  $-80^\circ\text{C}$ . It was subsequently resuspended in  $600 \mu\text{L}$  of  $100 \text{ mM}$  of phos-



**Fig. 1 – Representative pictures of Manila clams *Ruditapes philippinarum* of White (A), Liangdao Red (B) and Zebra (C) species with the shell length ranging from 3.4 to 3.8 cm.**

phate buffer ( $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ , including 0.5 mM TSP, pH 7.0) in  $\text{D}_2\text{O}$ . The mixture was vortexed and then centrifuged at  $3000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatant substance (550  $\mu\text{L}$ ) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

### 2.3. NMR spectroscopy

Extracts of adductor muscle tissue from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K). One-dimensional (1-D)  $^1\text{H}$  NMR spectra were obtained using a 11.9  $\mu\text{s}$  pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, with 128 transients collected into 16,384 data points (Fig. 2). Datasets were zero-filled to 32,768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All  $^1\text{H}$  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 tabulated chemical shifts (Fan, 1996; Viant et al., 2003) and some of them confirmed by the 2D NMR method,  $^1\text{H}$ - $^1\text{H}$  homonuclear correlation spectroscopy (COSY) (Fig. 3).

### 2.4. Spectral pre-processing and multivariate data analysis

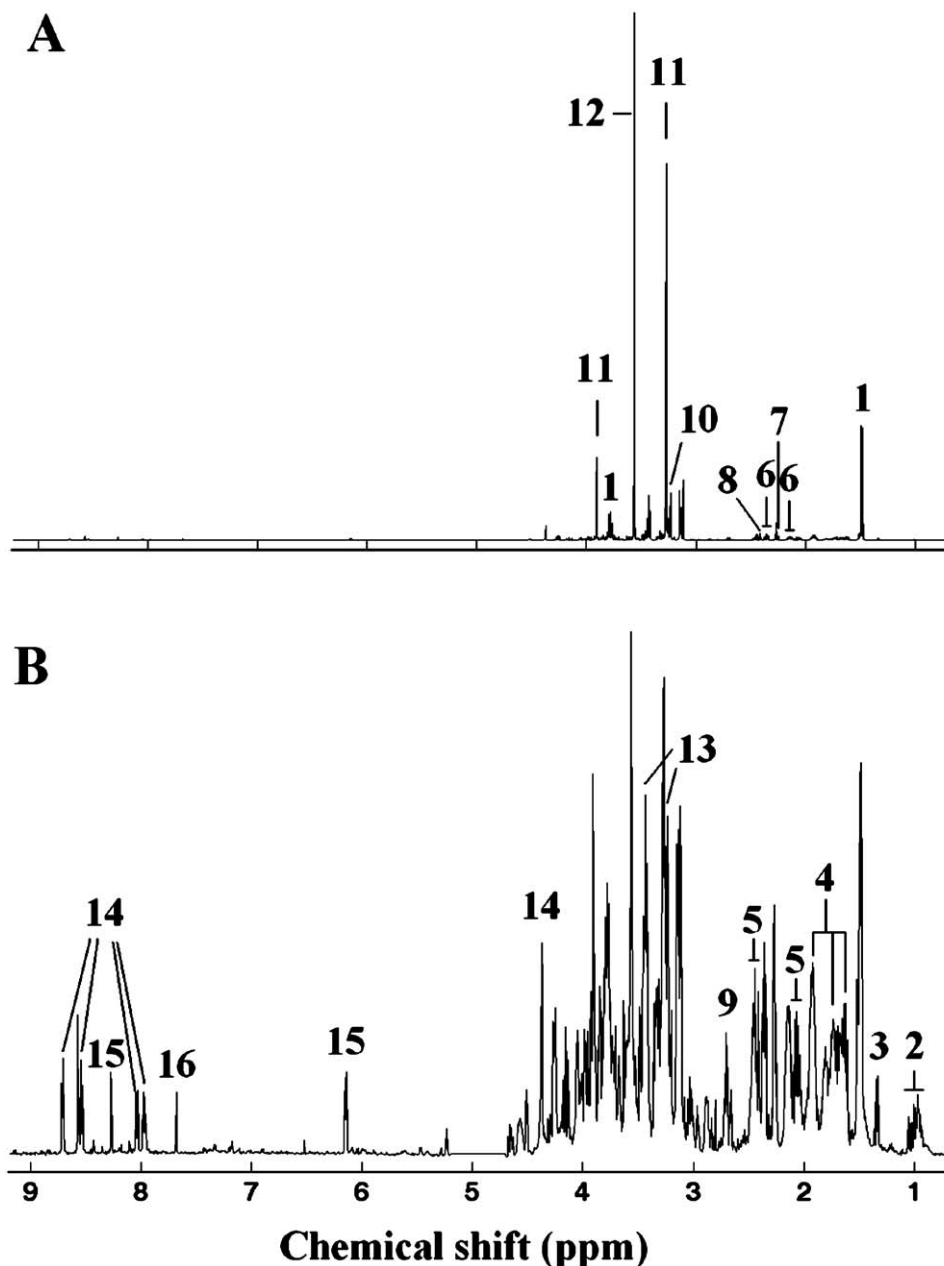
One dimensional proton NMR spectra were converted to a format for multivariate analysis using custom-written ProMetab software in Matlab (version 7.0; The MathsWorks, Natick, MA) (Purohit et al., 2004). Each spectrum was segmented into 0.005 ppm bins between 0.2 and 10.0 ppm with bins from 4.72 to 4.96 ppm (water) excluded from all the NMR spectra. Bins between 8.57 and 8.60 ppm, between 7.13 and 7.20 ppm, and between 7.67 and 7.69 ppm containing pH-sensitive NMR peaks were compressed into single bins. 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 (glog) with transformation parameter  $\lambda = 1.4 \times 10^{-9}$  (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 (Fig. 2).

The two well-developed pattern recognition techniques, Principal Components Analysis (PCA) and Partial Least-Squares Discriminant Analysis (PLS-DA), were used in this work for the separation of sample groups. PCA is an exploratory unsupervised pattern recognition (PR) method since it detects inherent variation within the dataset and takes no account of class membership. 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 was examined to identify the metabolites that contributed to the clusters (Xu, 2004). Partial Least Squares Discriminant Analysis (PLS-DA) is a supervised PR method to maximize the separation between the biological samples (Wold et al., 2001; Xu, 2004). In PLS-DA, the X matrix is the measured matrix, i.e., the NMR data, and the Y matrix is made of dummy variables consisting of ones and zeros that indicate the class for each treatment (Keun et al., 2003). The quality of the PLS-DA model was assessed using cross-validation with leave-one-out (Rubingh et al., 2006). A  $Q^2$  score of  $>0.08$  indicates that the model is significantly better than chance, while a score between 0.7 and 1.0 indicates that the model is highly robust (Eriksson et al., 2001). Data were mean-centered before PCA and PLS-DA using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA). One-way analysis of variance (ANOVA) was performed on the bin areas from NMR spectral peaks that were potentially significant for the separation between the sample groups to test the significance of corresponding metabolites.

## 3. Results and discussion

### 3.1. $^1\text{H}$ NMR spectroscopy of mussel tissue extracts

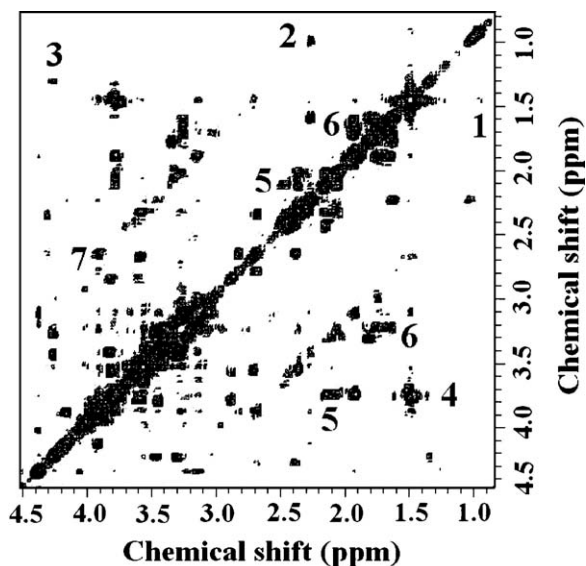
A representative  $^1\text{H}$  NMR spectrum (in both original and transformed forms) of mussel tissue extracts is shown in Fig. 2.



**Fig. 2** – A representative 1-dimensional 500 MHz <sup>1</sup>H NMR spectrum of adductor muscle tissue extracts from a White clam of control group in original (A) and generalized log transformed with a lambda value  $1.4 \times 10^{-9}$  (B) forms. Keys: (1) alanine, (2) branched chain amino acids: isoleucine, leucine and valine, (3) lactate, (4) arginine, (5) glutamate, (6) glutamine, (7) acetoacetate, (8) succinate, (9) aspartate, (10) acetylcholine, (11) betaine, (12) glycine, (13) taurine, (14) homarine, (15) ATP/ADP, and (16) unknown.

Overall, the original NMR spectrum (Fig. 2A) is dominated by one of the key organic osmolytes, betaine (3.25 and 3.91 ppm), which is ca. 10–100 times intense than other metabolites. Organic osmolytes such as betaine, homarine, and taurine are small organic molecules which maintain the osmotic balance in invertebrates. Those osmolytes can be actively accumulated in high salinity environments and released when the salinity decreases. Therefore, organic osmolytes play important physiological roles in osmotic regulation of invertebrates and hence were detected at relatively high levels in the clams (Preston,

2005). Hereby, the generalized log transformation was applied to the NMR bin areas to stabilize the technical variance and enhance the intensities of less abundant metabolites (e.g., homarine, Fig. 2B) which were likely crucial for the biological variance detection between the samples (Parsons et al., 2007). Several classes of metabolite were visibly observed in the <sup>1</sup>H NMR spectrum (Fig. 2B), including amino acids (e.g., alanine), energy storage compounds (e.g., ATP/ADP), Krebs cycle intermediates (e.g., succinate), and organic osmolytes (e.g., betaine).

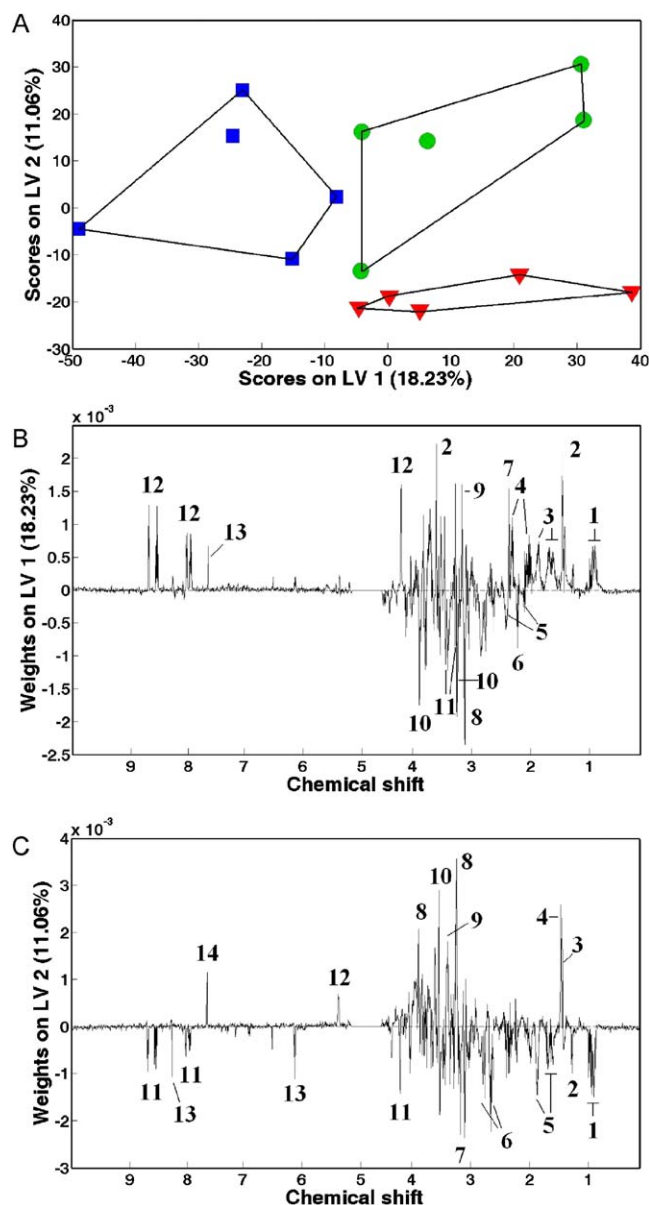


**Fig. 3** – A two-dimensional  $^1\text{H}$ - $^1\text{H}$  homonuclear correlation spectroscopy (COSY) NMR spectrum of adductor muscle extracts from a White clam used to confirm metabolite identification. Keys: (1) isoleucine, (2) valine, (3) lactate, (4) alanine, (5) glutamine, (6) arginine, and (7) aspartate.

### 3.2. Comparison between the metabolic profiles of adductor muscle extracts from untreated White, Liangdao Red and Zebra clams

Manila clam *R. philippinarum* is one of the representative species in the ‘Mussel Watch Program’ launched in China in 2004. Although Manila clams have been frequently studied in the marine and coastal environmental toxicology, the biological differences at molecular level between various species has often been ignored, which can induce distortion to the biological interpretation due to the different tolerance and sensitivity of clams to the environmental contaminants. In this limited work, we originally applied NMR-based metabolics to detect the metabolic differences between White, Liangdao Red and Zebra species of clams distributing mainly in Yantai population.

PCA was initially used to the NMR spectral matrix from 3 untreated species of clams to compare the metabolic profiles between species. However, no significant difference ( $p > 0.05$ ) was found between the groups using PCA (data not shown), hence the supervised technique PLS-DA was applied to classify the 3 groups of clam samples (Fig. 4). PLS-DA resulted in greater separation between the groups (Fig. 4A) than PCA and presented the differences between the metabolic profiles based on the weight plots which conveniently facilitated the identification of metabolic changes between the three species of clam groups (Fig. 4B, C). The LV (Latent Variable) score plots showed that NMR spectra from clams of Zebra species (blue squares) along negative LV1, while both Liangdao Red (green cycles) and White (inverted red triangles) species clustered mainly along positive LV1, and Liangdao Red and White species were located at positive and negative LV2, respectively (Fig. 4A).



**Fig. 4** – Partial Least-Squares Discriminant Analysis (PLS-DA) model showing (A) separations between untreated White ( $\nabla$ ), Liangdao Red ( $\bullet$ ) and Zebra ( $\blacksquare$ ) clam samples ( $Q^2 = 0.263$ ); and corresponding LV1 (B) and LV2 (C) weight plots showing the metabolic differences between the different species of clam. Keys in (B): (1) branched chain amino acids: isoleucine, leucine and valine, (2) unknown 1, (3) arginine, (4) glutamate, (5) glutamine, (6) acetoacetate, (7) succinate, (8) unknown 2, (9) acetylcholine, (10) betaine, (11) taurine, (12) homarine, and (13) unknown 3; and keys in (C): (1) branched chain amino acids: isoleucine, leucine and valine, (2) lactate, (3) alanine, (4) unknown 1, (5) arginine, (6) aspartate, (7) acetylcholine, (8) betaine, (9) taurine, (10) glycine, (11) homarine, (12) glycogen, (13) ATP/ADP and (14) unknown 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1 – List of the significantly abundant metabolites in 3 species of clam ( $p < 0.05^a$ ).**

Clam species	Abundant metabolites (ppm)
White	Branched-chain amino acid (0.94–1.04)
	Lactate (1.33)
	Arginine (1.73, 1.91)
	Aspartate (2.68, 2.81, 3.89)
	Acetylcholine (3.20)
	Homarine (4.37, 7.97, 8.04, 8.55, 8.72)
	ATP/ADP (6.15, 8.27, 8.54)
Liangdao Red	Branched-chain amino acids (0.94–1.04)
	Unknown 1 (1.51, 3.63, $R^2 = 0.90$ )
	Arginine (1.73, 1.91)
	Glutamate (2.10, 2.36)
	Succinate (2.41)
	Acetylcholine (3.20)
	Homarine (4.37, 7.97, 8.04, 8.55, 8.72)
Zebra	Unknown 2 (7.68)
	Glutamine (2.15, 2.45)
	Acetoacetate (2.25)
	Unknown 3 (3.15)
	Betaine (3.27, 3.91)
	Taurine (3.25, 3.43)

<sup>a</sup>  $p$  values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite.

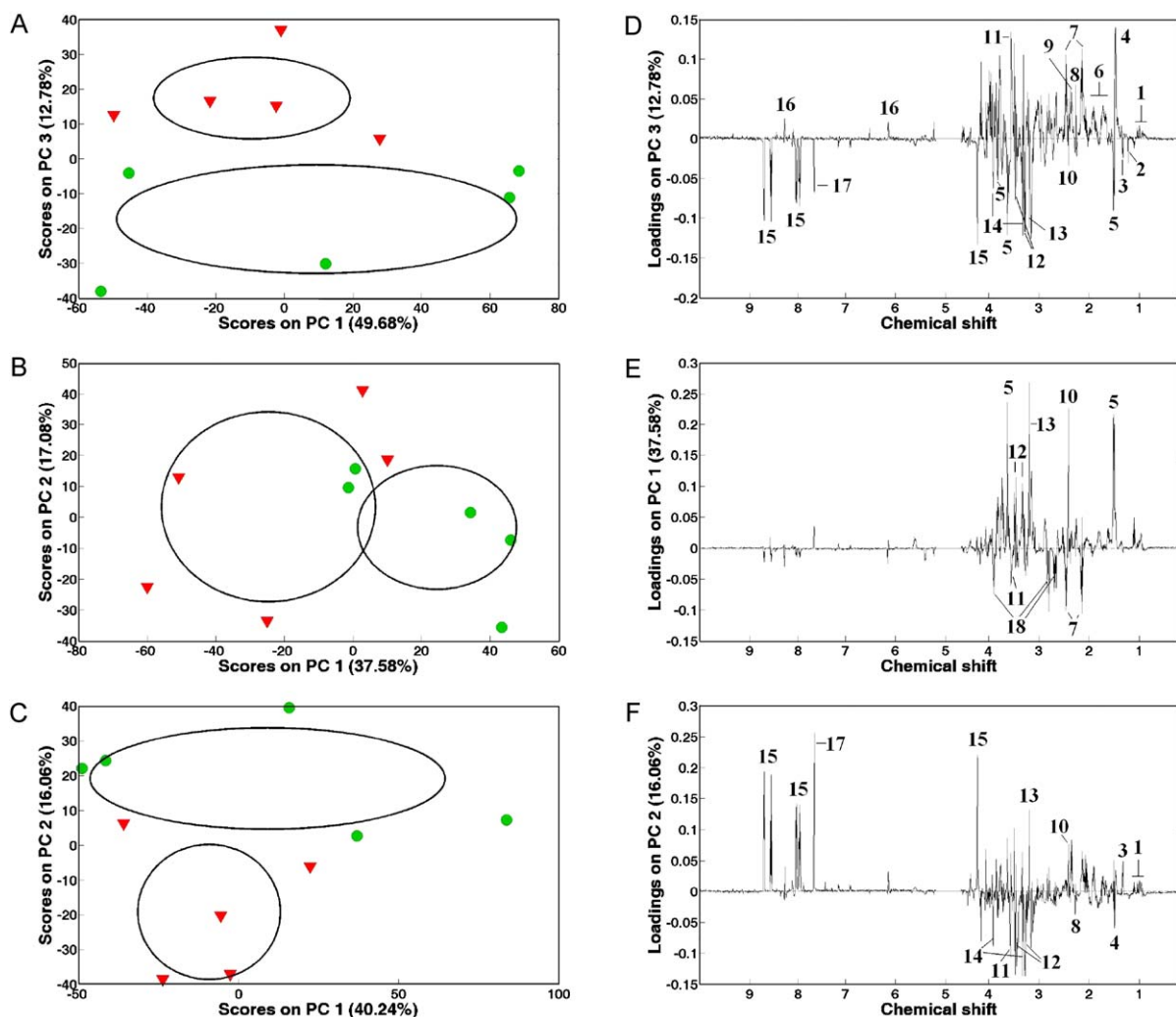
These indicated that the metabolic profiles of three species were inherently different to each other, and the detailed differences between the metabolic phenotypes were discovered using PLS-DA.

From the LV weight plots (Fig. 4B, C), the significant differences (the ratio of representative peak area of significant metabolites to total spectral area,  $p < 0.05$ ) between the metabolic profiles of 3 various species of clams were observed and are listed in Table 1. The most abundant metabolites in White clam samples were branched-chain amino acids (leucine, isoleucine and valine), lactate, arginine, aspartate, acetylcholine, homarine and ATP/ADP, while the metabolite profile of Zebra clam samples comprises high level of glutamine, acetoacetate, betaine, taurine and one unidentified metabolite (3.15 ppm). For Liangdao Red clam samples, the metabolite profile relatively exhibited high amounts of branched-chained amino acids, arginine, glutamate, succinate, acetylcholine, homarine and two unassigned metabolites (1.51 and 3.63 ppm,  $R^2 = 0.90$ , 7.68 ppm). Although these metabolic differences between the species are related to complex metabolic pathways and physiological regulations, all the three species of clam are derived from the same species, the Manila clam *R. philippinarum* sharing the similar genotypic milieu. Therefore the differences of phenotypic fingerprinting (e.g., metabolic differences) between various species of clam might be generated from the differential gene expressions and consequent amounts of enzymes related to the corresponding metabolic pathways such as osmotic regulations. However, further studies on the mechanisms of metabolic differences between the species are necessary.

### 3.3. Comparisons between the metabolic responses of adductor muscle from White, Liangdao Red and Zebra clams exposed to $Hg^{2+}$

The metabolic differences between White, Liangdao Red and Zebra species were found successfully, which suggested that a purebred species of clam should be studied in the marine or coastal environmental toxicology to minimize the biological variations induced by various species. Therefore, a further study should be performed on the sensitivity of various species of clam to the environmental contaminant to identify a sensitive species of clam for the heavy metal monitoring. In this work, the inorganic mercury ( $Hg^{2+}$ ) was used to test the metabolic responses in the three species of clams since mercury has become one of the highest ecologically risky contaminants in the Bohai coastal environment due to its high toxicity. Inorganic mercury is a persistent toxic and hazardous heavy metal that can rapidly associate with colloid and other ultrafine materials in water and therefore presents a risk to organisms such as filter-feeders, e.g. Manila clam by binding highly to sulfhydryl that are abundant in proteins and polypeptides of organism (Clarkson, 1997). Thus, it is often found in cells and tissues bound with thiol-containing proteins and small-molecular weight thiols such as cysteine and glutathione (GSH). In addition, mercury can activate free radicals that induce lipid, protein, and DNA oxidation (Lund et al., 1993; Clarkson, 1997).

PCA was performed on the  $^1H$  NMR spectral data sets generated from the control and  $Hg^{2+}$ -exposed groups of clams from White, Liangdao Red and Zebra species, respectively. From the PC scores plots (Fig. 5A–C), the separations between the control (inverted red triangles) and exposed (green cycles) were obviously observed ( $p < 0.05$ ), which suggested the significant metabolic changes in adductor muscle tissues of clams induced by  $Hg^{2+}$  exposure after 48 h. The metabolic profiles from White species of clams showed significantly increase in lactate, succinate, taurine, acetylcholine, betaine, homarine and 2 unidentified metabolite (1.23 and 1.51, 3.63 ppm,  $R^2 = 0.91$ ) and decrease of branched-chain amino acids (leucine, isoleucine and valine), alanine, arginine, glutamine, glutamate, acetoacetate, glycine, ATP/ADP and an unknown metabolite at 7.68 ppm (Table 2, Fig. 5D). The distinguishable metabolic changes caused by  $Hg^{2+}$  exposure in Liangdao Red clam muscles included the increased succinate, taurine, acetylcholine, and the unknown metabolite at 1.51 and 3.63 ppm, together with the decreased glutamine, glycine and aspartate (Table 2, Fig. 5E). For the Zebra species, the elevated branched-chain amino acids, lactate, succinate, acetylcholine, homarine and the unknown metabolite at 7.68 ppm were found as well as the declined alanine, acetoacetate, glycine and taurine (Table 2, Fig. 5F). The elevation of acetylcholine was commonly found in the  $Hg^{2+}$ -exposed groups of clams from all the three species. Acetylcholine is a neural transmitter that can be degraded to choline in cholinergic synapses and neuromuscular junctions by acetylcholinesterase (AChE) (Matozzo et al., 2005). Indeed,  $Hg^{2+}$  is a known neurotoxic substance to the animals by interrupting the nervous transmission. In some studies, the measurement



**Fig. 5** – Principal Components Analysis (PCA) on the  $^1\text{H}$  NMR spectra of both control ( $\blacktriangledown$ ) and  $\text{Hg}^{2+}$ -exposed ( $\bullet$ ) clams from White (A), Liangdao Red (B) and Zebra (C) species, and corresponding PC loadings plots, (D)–(F) showing the metabolic differences between the control and  $\text{Hg}^{2+}$ -exposed clams after 48 h of exposure. Ellipses represent means  $\pm$  standard deviation of PC scores for the various groups. Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) unknown 1, (3) lactate, (4) alanine, (5) unknown, (6) arginine, (7) glutamine, (8) acetoacetate, (9) glutamate, (10) succinate, (11) glycine, (12) taurine, (13) acetylcholine, (14) betaine, (15) homarine, (16) ATP/ADP, and (17) unknown 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of AChE activity was demonstrated useful as a biomarker of neurotoxic compounds in aquatic organisms and has been successfully applied to the monitoring of neurotoxic contaminations (Cajaraville et al., 2000; Matozzo et al., 2005). For example, Matozzo et al. reported that clams collected at Marghera, a highly polluted area, showed lower AChE activity than that of animals from both Campalto and Poveglia (pristine sites) indicating that enzyme inhibition was due to exposure to neurotoxic substances. As a matter of fact, the inhibition of AChE is followed by acetylcholine accumulation, therefore, the elevation of acetylcholine could be a metabolic biomarker of neurotoxicity of  $\text{Hg}^{2+}$  induced by in clams.

The levels of succinate (a key intermediate of Kreb's cycle) were increased in the muscle samples from all the three  $\text{Hg}^{2+}$  exposed species of clams as well. The accumulation of

succinate is a clear biomarker of facultative anaerobiosis in molluscs (De Zwaan et al., 1976). Thus, the increased succinate in the clam muscle implied the perturbation of energy metabolism, especially with the decrease of ATP/ADP in the White clams. Levels of lactate increased in White and Zebra clams after mercury exposure suggesting that gluconeogenesis was inhibited in White and Zebra clams. Lactate is related to the reduced use of metabolites in the citric acid cycle and to an increase in anaerobic cell energy metabolism, which indicated an enhancement in anaerobic metabolism like the elevation of succinate in all the three species of clam. These normally result from conditions where the activation of glycolysis exceeds that of oxidative phosphorylation (i.e., extreme exercise) or from muscle activity where energy demand exceeds energy supply. Also, the increased lactate in White and Zebra clams indicated that these species of clam

**Table 2 – Significantly up- or down-regulated metabolites ( $p < 0.05^a$ ) from 3 species of clams with acute (48 h) mercury exposure.**

Clam species	Metabolites increased in exposed samples (ppm)	Metabolites decreased in exposed samples (ppm)
White	Unknown 1 (1.23) Lactate (1.33) Unknown 2 (1.51, 3.63) Succinate (2.41) Taurine (3.25, 3.43) Acetylcholine (3.20) Betaine (3.27, 3.91) Homarine (4.37, 7.97, 8.04, 8.55, 8.72)	Branched-chain amino acids (0.94–1.04) Alanine (1.48) Arginine (1.73, 1.91) Glutamine (2.15, 2.45) Acetoacetate (2.25) Glutamate (2.10, 2.36) Glycine (3.57) ATP/ADP (6.15, 8.27, 8.54) Unknown 3 (7.68)
Liangdao Red	Unknown 2 (1.51, 3.63) Succinate (2.41) Taurine (3.25, 3.43) Acetylcholine (3.20)	Glutamine (2.15, 2.45) Glycine (3.57) Aspartate (2.68, 2.81, 3.89)
Zebra	Branched-chain amino acids (0.94–1.04) Lactate (1.33) Succinate (2.41) Acetylcholine (3.20) Homarine (4.37, 7.97, 8.04, 8.55, 8.72) Unknown 3 (7.68)	Alanine (1.48) Acetoacetate (2.25) Glycine (3.57) Taurine (3.25, 3.43)

<sup>a</sup>  $p$  values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite.

were sensitive to the mercury exposure in the response of energy metabolism. Arginine is the end product of phosphoarginine + ADP → arginine + ATP, in which phosphoarginine is the primary phosphagen in invertebrates, serving as both a spatial and temporal energy buffer in tissues with high-energy demands. This process is catalyzed by arginine kinase, which exchanges a phosphate from phosphoarginine to ADP, thus maintaining a stable ATP concentration (Viant et al., 2001). In this study, the depletion of arginine and ATP/ADP in the White clams might be the biomarker of disorder of energy metabolism. However, Viant et al. (2001) reported contrarily that the arginine and ATP would be elevated with a depletion of phosphoarginine in the gill tissue of abalone exposed to 66  $\mu\text{g L}^{-1}$  copper after 8 h of waterborne exposure in their study, this could be accounted for the various toxicological mechanisms between copper and mercury. The unique metabolic changes in the arginine and ATP/ADP suggested that White clam was the most sensitive species to the mercury exposure.

Betaine, taurine, glycine, homarine, and homarine are known as organic osmolytes in the marine bivalves. The metabolic changes in these metabolites showed the disturbance of  $\text{Hg}^{2+}$  to the osmolarity balance. However, the metabolic changes of organic osmolytes (Table 2) were distinctive between the three species, which should be caused by different mechanisms and needs further study. On the basis of changed organic osmolytes, White clam exhibited a more sensitive response of osmolarity to the mercury exposure, since there were 4 organic osmolytes up- or down-regulated than that of Liangdao Red (2 osmolytes) and Zebra species (3 osmolytes).

Specially, the decrease in the levels of glutamine and glutamate (a transmitter of central nervous system) were uniquely found in the muscle samples of White clam. The metabolism of glutamate and glutamine involves a series of pathways, in

which glutamate can be converted to glutamine by glutamate synthetase. Thus, the decrease of glutamate and glutamine could be the neurotoxic effects induced by mercury in the White clams, which was consistent with the elevation of acetylcholine.

In conclusion, this limited study focused on the differences of metabolic profiles from White, Liangdao Red and Zebra clams and the metabolic responses of various species of clam to the acute mercury exposure to determine a suitable species of clam as bioindicator for heavy metal monitoring and environmental toxicology. Although the White, Liangdao Red and Zebra species of clam are all derived from the same species, Manila clam *R. philippinarum*, the metabolic differences were found by NMR-based metabolomics. The most abundant metabolites in White clam samples were branched-chain amino acids (leucine, isoleucine and valine), lactate, arginine, aspartate, acetylcholine, homarine and ATP/ADP, while the metabolite profile of Zebra clam samples comprises high level of glutamine, acetoacetate, betaine, taurine and one unidentified metabolite. For Liangdao Red clam samples, the metabolite profile relatively exhibited high amount of branched-chained amino acid, arginine, glutamate, succinate, acetylcholine, homarine and two unassigned metabolites. After 48 h exposure of 20  $\mu\text{g L}^{-1}$   $\text{Hg}^{2+}$ , the metabolic profiles showed significantly distinguishable differences between three species of clam, which respectively included increased lactate, betaine and homarine metabolites and decreased arginine, and ATP/ADP in White clams, and declined aspartate and glutamine in Liangdao Red clams, and elevated branched-chain amino acids and reduced taurine in Zebra clam samples. Overall, our findings suggested that White clams could be the preferable bioindicator for the metal pollution monitoring because of the more sensitive metabolic changes due to the  $\text{Hg}^{2+}$  exposure compared with other two (Liangdao Red and Zebra) species of clams. However, further studies are needed to



examine, (1) the sensitivity test of clam muscle tissues to other environmental contaminants including other heavy metals, and (2) the sensitivity test of other clam tissues such as digestive gland or gill to heavy metals to determine a sensitive tissue type for the metal monitoring.

### Conflict of interest

Nothing declared.

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