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Evaluation of metal pollution-induced biological effects in Chinese shrimp *Fenneropenaeus chinensis* by NMR-based metabolomics

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ABSTRACT

Metal pollution in Laizhou Bay along the Bohai Sea in China has been posing a risk on fishery species and hence may affect seafood quality. In this work, shrimps *Fenneropenaeus chinensis* were sampled from three sites, namely, a reference (site 6334) and two metal-polluted (sites 6262 and 7262) sites, located in Laizhou Bay. The metal concentrations in shrimp muscle tissues were tested using the ICP-MS technique. The Cr and Cu concentrations were the highest in the shrimp samples from site 7262, exceeding the national seafood safety standard II, and the As concentration was much higher than the national seafood safety standard III. NMR-based metabolomics indicated that metal pollution induced oxidative and immune stresses, damaged the muscular structure, and disrupted energy metabolism in shrimps at sites 6262 and 7262, in particular disturbed osmotic regulation in shrimps at site 7262. Glycine and serine could serve as biomarkers for Cd in *F. chinensis*.

Metal pollution in the marine environment has obvious spatial distribution characteristics resulting from industrial sources along the coastline of the Bohai Sea. Gao et al. (2014) reported that Cd and Cu were the main metal contaminants in Jinzhou Bay and Liaodong Bay, respectively. Our recent studies reported significant Cd and As pollution in the edible shrimp *Crangon affinis* sampled from Laizhou Bay and the Yellow River Estuary (Ji et al., 2016; Xu et al., 2016). The discharge of industrial effluents from some coal-mining and gold-mining areas in the industrial cities along Laizhou Bay is an important source for the huge amounts of metals in the coast and marine environment (Liang et al., 2011; Liu et al., 2016). Therefore, metal pollution poses a threat to the quality of shrimp species of economic importance in the Bohai Sea and consequently may induce adverse effects in these species (Li et al., 2015).

The Chinese shrimp *Fenneropenaeus chinensis* is one of the most important species of marine fishery and distributed along the coastal environment in the Bohai Sea (Li et al., 2018b). Because of its delicious flavor, *F. chinensis* is widely consumed by people, leading to its substantial economic value for fishery. As reported, the maximum yield of *F. chinensis* once achieved 50,000 tons (Song et al., 2018). Currently, the heavy metal pollution in the Bohai Sea is a concerning issue with regard to the security of seafood. Usually, the muscle tissues of marine animals can accumulate lower levels of metals than other tissues such as those in the digestive gland. As the muscle of *F. chinensis* is the main edible part, the potential risk of metal accumulation in the muscle tissue of this shrimp species should not be ignored. In addition, there is a need to characterize the biological effects induced by metal pollution.

In recent years, the newly established –omics approaches (genomics, transcriptomics, proteomics, and metabolomics) have been extensively applied in the areas of environmental science (Cappello et al., 2013; Ji et al., 2019; Song et al., 2016). As the end products of metabolism, metabolites are good indicators of the physiological and biochemical status in organisms (Jones et al., 2008; Viant et al., 2003). Therefore, the metabolic perturbations could be used to interpret the biological effects induced by exogenous factors such as pollutants (Viant et al., 2003). Usually, modern analytical techniques such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are used to detect minor metabolic alterations in stress-exposed tissue samples to elucidate the biological effects induced by environmental pollutants (Cappello et al., 2013, 2018; Caricato et al., 2019; Fasulo et al., 2012; Kwon et al., 2012; Maisano et al., 2017; Vignet et al., 2019). In the present study, inductively coupled plasma mass spectrometry (ICP-MS) was used to analyze the metal contamination in the Chinese shrimps *F. chinensis* collected from metal-polluted sites

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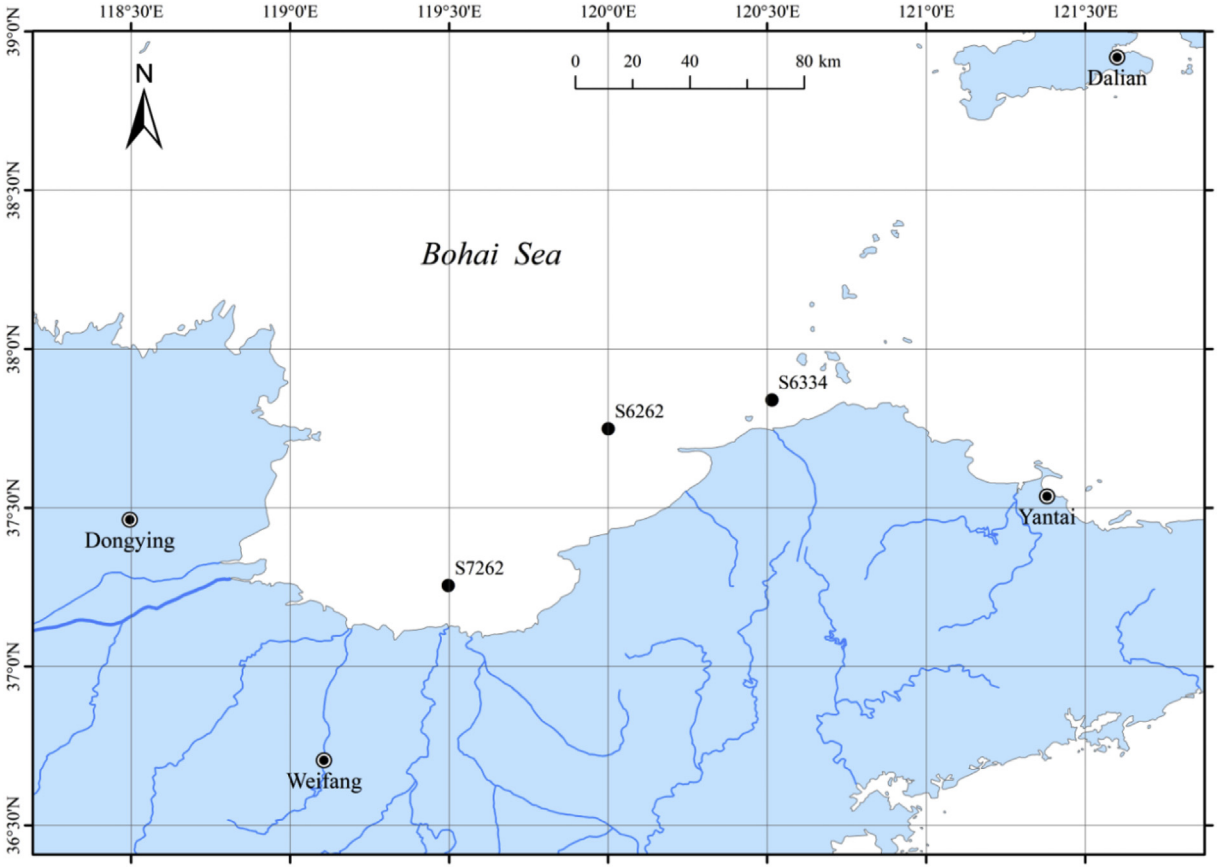

Fig. 1. Map showing the locations of sampling sites in Laizhou Bay, Bohai Sea, China. 1. S6334: 37°50′0″ N, 120°30′0″ E; 2. S6262: 37°45′0″ N, 120°0′0″ E; 3. S7262: 37°15′0″ N, 119°30′0″ E.

along Laizhou Bay, and NMR-based metabolomics was employed to characterize metabolic alterations to elucidate the biological effects in shrimp muscle tissues induced by metal pollution from industrial discharge (Liang et al., 2011; Liu et al., 2016).

The shrimps *F. chinensis* were sampled from site 6334 (S6334: 37°50′0″ N, 120°30′0″ E), site 6262 (S6262: 37°45′0″ N, 120°0′0″ E), and site 7262 (S7262: 37°15′0″ N, 119°30′0″ E) located along Laizhou Bay along the Bohai Sea in August 2017 (Fig. 1). S6334 was relatively clean and therefore selected as the reference site (Liang et al., 2011). At least eight individual shrimps of similar size (~15 cm) were sampled from each site, and the muscle tissues were dissected and immediately flash frozen in liquid nitrogen. After transportation to our laboratory, the muscle tissues of the shrimps were stored at −80 °C before metal analysis and metabolite extraction. All the experimental procedures for sampling were strictly conducted according to Hines et al. (2007).

Metabolites were extracted from the muscle tissues of shrimps (n = 8) using the methanol/water/chloroform solvent system (Zhang et al., 2011). Briefly, the muscle tissue (~100 mg) was ground into powder in liquid nitrogen and then homogenized using a high-throughput homogenizer (Precellys 24, Bertin Technologies, France). The homogenized shrimp muscle sample was extracted in a solvent system that included methanol (4 mL/g), water (0.85 mL/g), and chloroform (2 mL/g). After shaking and centrifuging (3000 *g*) for 5 min at 4 °C, the upper layer (methanol/water) containing metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The dried extract was resuspended in 600 μL of 100 mM phosphate buffer (Na_2HPO_4 and NaH_2PO_4 with 0.5 mM TSP, pH 7.0) in D_2O. The mixture was vortexed and then centrifuged (3000 *g*) for 5 min at 4 °C. Finally, 550 μL was accurately transferred into an NMR tube (5 mm) for ^{1}H NMR spectroscopy measurement with a Bruker AV 500 NMR spectrometer at 25 °C (Zhang et al., 2011). The details of NMR parameters have been described by Zhang et al. (2011).

After phase correction, baseline correction, and calibration (TSP at 0.0 ppm), the ^{1}H NMR spectra were converted in batch to a data matrix using ProMetab software in MatLab (V7.0, The MathWorks, Natick, MA, USA) (Viant et al., 2003). Each spectrum was divided into bins between 0.2 and 10.0 ppm, with each bin width at 0.005 ppm. The bins from water peak between 4.70 and 5.20 ppm were deleted from the ^{1}H NMR spectra. The total spectral area of the remaining bins was normalized to unity and generalized log-transformed using a transformation parameter λ (= 1.0×10^{-8}) to stabilize the variance across the spectral bins (Zhang et al., 2011).

Before multivariate data analysis, mean-centering was applied for the preprocessing of NMR spectral data. Principal component analysis (PCA) was adopted to separate the groups of shrimp muscle samples collected from three sites (S6334, S6272, and S7262) in Laizhou Bay. Then, supervised multivariate data analysis methods, partial least squares-discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were performed on the datasets to detect the metabolites significantly induced by metal pollution. The classifications and spectral variables contributing to the classifications were shown in the score plots and corresponding loading plots, respectively. The model coefficients were computed from the coefficients incorporating the weight of the variables to enhance the interpretability of the model. Detailed information of data analysis has been described by Feng et al. (2013). Metabolites were identified using Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada) software.

The shrimp muscle tissue samples (n = 8) were weighed and digested in concentrated HNO_3 at 80 °C for 12 h. Metal concentrations in shrimp muscle samples were determined using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700x). On the basis of

recent investigations, the metals/metalloids Pb, Cr, Mn, Fe, Co, Ni, Cu, As, Zn, Se, and Cd were considered potential metal pollutants in the sampling sites (Gao et al., 2014; Ji et al., 2016; Xu et al., 2016). Therefore, the concentrations of these metals/metalloids were quantified in the shrimp samples. Internal standards (Sc, Ge, In, and Bi) were selected to correct the sensitivity drift and matrix effect. A quality control sample was repeatedly measured after every 10 samples. The recovery of the analyzed metals from the standard reference material (SRM 1566b, oyster tissue) was within 10% deviation from the certified values, except for Cr, whose certified concentration was not available. Metal concentrations were expressed as mean ± standard deviation (S.D.) and subjected to Student *t*-test using Minitab 15 (Minitab, PA, USA). *P* values of less than 0.05 were considered statistically significant.

The Chinese shrimps *F. chinensis* have delicious flavor and are a good source of nutrition, leading to substantial consumption by local people residing along the Bohai Sea. Therefore, this species has a great economic value in marine aquaculture (Song et al., 2018). However, metal pollution in the Bohai Sea resulting from industrial discharges has been a concerning issue (Gao et al., 2014; Li et al., 2018a). Unfortunately, there is little information regarding the metal concentrations in the muscle tissues of *F. chinensis* in the Bohai Sea. In this work, the metal/metalloid concentrations were measured in muscle tissues of shrimp *F. chinensis* collected from three sites in Laizhou Bay along the Bohai Sea (Table 1). Further, a simple equation was used to compare the integrated metal contamination (IMC) in these three sampling sites (Liu and Wang, 2012):

$$\text{Integrated metal contamination} = \sum_{i=0}^{m} C^{i}_{\text{contaminated}} - C^{i}_{\text{clean}}$$

where $C^{i}_{\text{contaminated}}$ is the concentration of the i*th* metal/metalloid in a contaminated shrimp sample, and C^{i}_{clean} is the reference value for the i*th* metal/metalloid in an uncontaminated shrimp sample. The reference value was determined from the shrimps collected from the relatively clean site S6334. m is the number of all metals/metalloids that a shrimp was simultaneously exposed to, i.e., m = 8 in this work. The IMC values indicated that the shrimp samples collected from S7262 had the highest metal content, while the samples collected from S6262 had a moderate metal content. As S7262 is located in the inner part of Laizhou Bay, seawater exchange in this site is slower than that in S6262. This indicated that the metal pollution at S7262 was more severe than that at S6262 because of the presence of As, Cr, Cu, and Cd in the industrial discharge from coal-mining and gold-mining factories in surrounding cities along Laizhou Bay (Li et al., 2019; Liang et al., 2011; Liu et al., 2016). As shown in Table 1, the highest concentrations of Cr, Cu, and As were found in the shrimp samples collected from S7262. The average concentrations of Cr and Cu in the shrimp samples collected from S7262 exceeded the national seafood safety standard II (National Standard of PR China, 2005). More severely, the average concentration of As (~11 μg/g wet weight) in the shrimp samples collected from S7262 was much higher than the national seafood safety standard III (8.0 μg/g wet weight). Although the average concentration of Cd did not exceed the safety criteria, the shrimp samples collected from S6262 presented the highest Cd concentration that was approximately 3 times higher than that from the reference site (S6334). These findings suggest us to pay more attention to the metal contamination and seafood security in the shrimp *F. chinensis*. In addition, the biological effects induced by the metal pollution need to be characterized in the shrimp *F. chinensis*.

A representative ^{1}H NMR spectrum of muscle tissue extracts of shrimps collected from S6334is shown in Fig. 2. A total of 37 metabolites were identified. These metabolites could be classified into osmolytes (dimethylglycine, trimethylamine N-oxide, betaine, taurine, homarine, and hypotaurine), energy storage compounds (glucose, glycogen, and ATP), organic acids (lactate, succinate, and fumurate), and

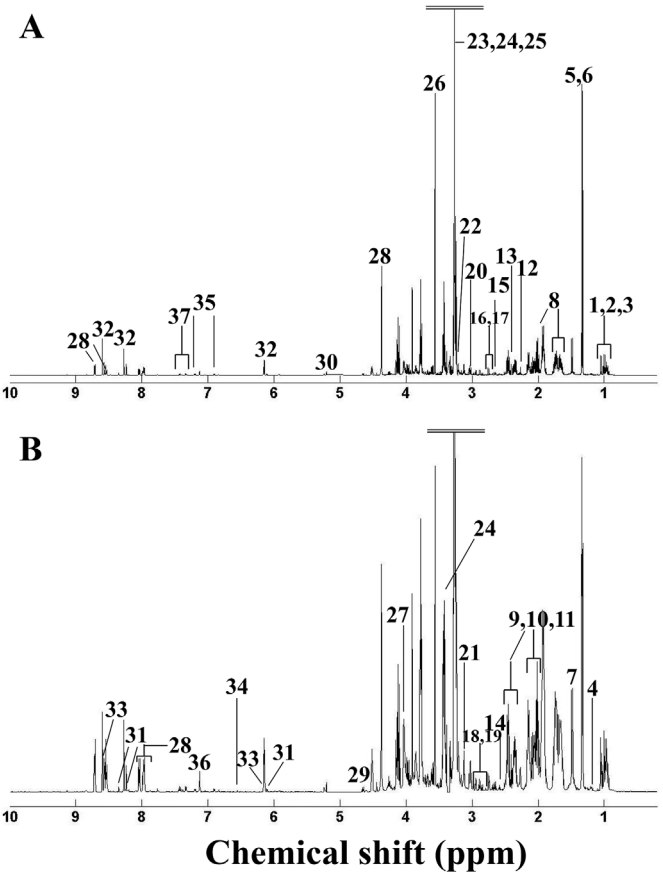

Fig. 2. A representative 1-dimensional 500 MHz ^{1}H NMR spectrum of muscle tissue extracts of shrimp *Fenneropenaeus chinensis* from the reference site (S6334) in the original (A) and generalized transformed (B) forms. **Keys:** (1) leucine, (2) isoleucine, (3) valine, (4) 3-hydroxybutyrate, (5) lactate, (6) threonine, (7) alanine, (8) arginine, (9) proline, (10) glutamate, (11) glutamine, (12) acetoacetate, (13) succinate, (14) methionine, (15) hypotaurine, (16) unknown 1 (2.74 ppm), (17) unknown 2 (2.76 ppm), (18) asparagine, (19) dimethylglycine, (20) lysine, (21) malonate, (22) phosphocholine, (23) trimethylamine N-oxide, (24) taurine, (25) betaine, (26) glycine, (27) serine, (28) homarine, (29) β-glucose, (30) α-glucose, (31) inosine, (32) AMP, (33) ATP, (34) fumarate, (35) tyrosine, (36) histidine, and (37) phenylalanine.

Table 1
Metal/metalloid concentrations in the muscle tissues from shrimp *Fenneropenaeus chinensis* sampled from three sampling sites, namely, the reference site (S6334) and the two metal-polluted sites (S6262 and S7262), in Laizhou Bay along the Bohai Sea.

Metal/metalloid concentration [a]	Sampling site		
	S6334	S6262	S7262
Pb	0.070 ± 0.037	0.057 ± 0.020	0.044 ± 0.016
Cr	1.967 ± 0.193	1.951 ± 0.203	2.510 ± 0.397**
Mn	0.954 ± 0.432	0.587 ± 0.298	0.559 ± 0.172
Fe	46.925 ± 28.577	36.648 ± 38.353	28.193 ± 26.247
Co	0.019 ± 0.010	0.026 ± 0.019	0.015 ± 0.007
Ni	0.102 ± 0.053	0.098 ± 0.034	0.123 ± 0.070
Cu	15.164 ± 6.474	22.676 ± 15.373	28.681 ± 17.423*
Zn	23.440 ± 2.400	22.480 ± 3.048	25.966 ± 1.850
As	6.133 ± 1.889	6.487 ± 1.658	11.178 ± 2.945**
Se	2.858 ± 0.873	2.673 ± 0.670	4.842 ± 1.169
Cd	0.006 ± 0.001	0.016 ± 0.008**	0.010 ± 0.004**

* ($P < 0.05$) and ** ($P < 0.01$) indicate the significant differences in metal concentrations between the reference site (S6334) and the metal-polluted sites (S6262 and S7262) (Student *t*-test).

[a] Data are shown as mean ± standard deviation ($n = 8$). Values are presented as μg/g wet weight.

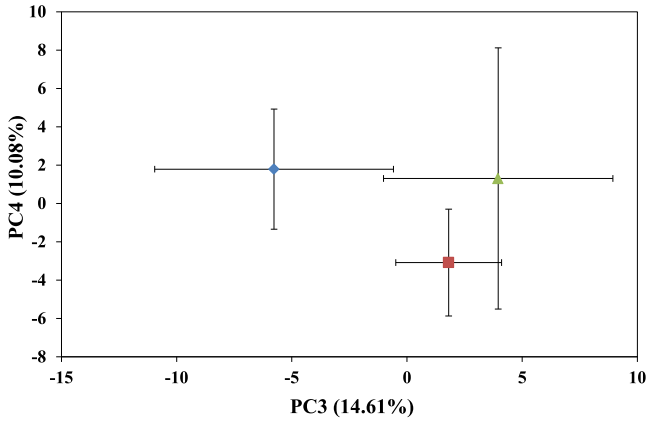

Fig. 3. Score plot of principal component analysis (PCA) along the PC3 and PC4 axes generated from ^{1}H NMR spectra of the muscle tissue extracts from shrimps *F. chinensis* sampled from S6334 (♦), S6262 (■), and S7262 (▲).

amino acids (isoleucine, leucine, valine, alanine, arginine, proline, glutamate, methionine, glycine, serine, etc.) (Cappello et al., 2017; Ji et al., 2016; Xu et al., 2016). PCA was primarily performed on the NMR spectral dataset of the shrimp samples collected from three sampling sites (S6334, S6262, and S7262), and this resulted in clear separation into the reference group (S6334) and metal pollution-exposed groups (S6262 and S7272) (Fig. 3). In detail, the metal pollution-exposed groups were located along the positive PC3 axis (14.61% variation). In addition, the S6262 group was also significantly ($P < 0.05$) separated along the negative PC4 axis (10.08% variation). The significant separations revealed that there were significant metabolic differences between the reference and metal pollution-exposed groups. Then, O-PLS-DA was applied to the NMR spectral data of the reference group (S6334) and metal pollution-exposed groups (S6262 and S7262) (Fig. 4).

Table 2
Relative change trends of metabolites in shrimp muscle samples from metal-polluted sites (S6262 and S7262) when compared with those from the reference site (S6334).

Metabolites	Typical chemical shift (ppm)	Metal-polluted sites	
		S6262	S7262
valine	1.05 (d)	↓	↓
isoleucine	1.00 (d)	↓	-
leucine	0.94 (d)	↓	-
glutamine	2.14 (m)	↓	-
asparagine	2.92 (ABX)	↓	-
lysine	3.03 (t)	↓	-
glycine	3.56 (s)	↑	↑
serine	3.97 (m)	↓	-
ATP	6.15 (d)	↑	-
lactate	1.33 (d)	-	↑
arginine	1.70 (m)	-	↑
proline	2.02 (m)	-	↑
trimethylamine N-oxide	3.26 (s)	-	↓
malonate	3.13 (s)	-	↑
phosphocholine	3.22 (s)	-	↑
homarine	4.37 (s)	-	↓
inosine	6.11 (d)	-	↑

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

The score plots generated by O-PLS-DA could present pairwise

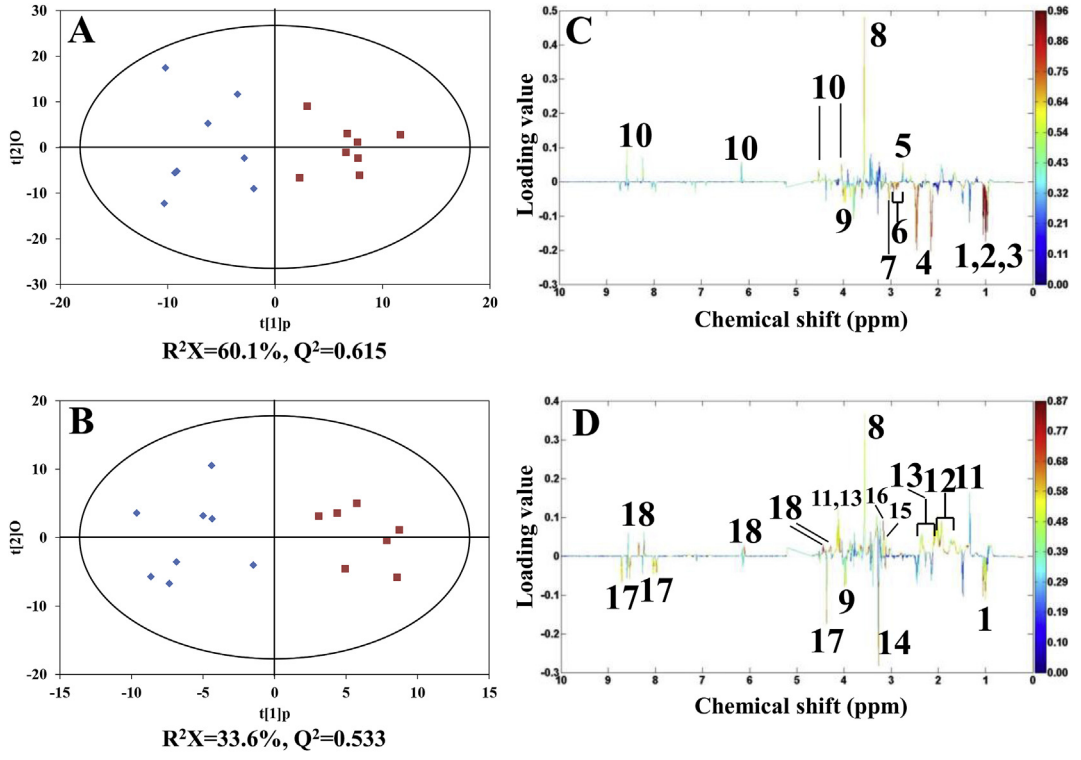

Fig. 4. O-PLS-DA scores derived from ^{1}H NMR spectra of the muscle tissue extracts from shrimp *F. chinensis* sampled from the reference site (♦) and the metal-polluted site (■), (A) S6334 vs. S6262 and (B) S6334 vs. S7262 and the corresponding coefficient plots (C) and (D). The color map shows the significance of metabolite variations between the two classes (reference and metal-polluted sites). Peaks in the positive direction indicate metabolites that are more abundant in the metal-polluted groups. Consequently, metabolites that are more abundant in the samples collected from the reference site are presented as peaks in the negative direction. **Keys:** (1) valine, (2) isoleucine, (3) leucine, (4) glutamine, (5) unknown 1 (2.74 ppm), (6) asparagine, (7) lysine, (8) glycine, (9) serine, (10) ATP, (11) lactate, (12) arginine, (13) proline, (14) trimethylamine N-oxide, (15) malonate, (16) phosphocholine, (17) homarine, and (18) inosine. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

classifications between the reference and metal pollution-exposed groups. As shown in Fig. 4A and B, the robust classifications were found by O-PLS-DA between the reference group (S6334) and the metal pollution-exposed groups (S6262 and S7262), with reliable Q^2 values (> 0.5). Significant metabolic differences between the reference group (S6334) and the metal pollution-exposed groups (S6262 and S7262) are shown in the corresponding loading plots in Fig. 4C and D and summarized in Table 2.

As shown in the loading plot of O-PLS-DA (Fig. 4C) and in Table 2, when compared with the metabolic profiles of shrimp samples collected from the reference site (S6334), the shrimp samples collected from S6262 had higher levels of glycine and ATP and lower levels of branched-chain amino acids (BCAAs; valine, isoleucine, and leucine), glutamine, asparagine, lysine, and serine, and the shrimp samples collected from S7262 (Fig. 4D and Table 2) had higher levels of glycine, lactate, arginine, proline, malonate, phosphocholine, and inosine and lower levels of valine, trimethylamine N-oxide, serine, and homarine.

Among these metabolic differences, the alteration of valine, serine, and glycine was common in the shrimp samples collected from both S6262 and S7262. Serine hydroxymethyltransferase catalyzes the reversible cleavage of serine to glycine (Vatsyayan and Roy, 2007). In yellow perch *Perca flavescens*, Cd exposure could significantly up-regulate the expression level of hydroxymethyltransferase, implying the enhanced conversion of serine to glycine (Bougas et al., 2013). This finding suggested a specific protection mechanism for cells against Cd-induced oxidative stress (Stocker et al., 2003). In this work, glycine and serine levels were increased and decreased, respectively, which indicated the enhanced conversion of serine to glycine in the shrimp samples collected from both metal pollution sites, S6262 and S7262. Interestingly, the Cd concentrations in the shrimp samples collected from both metal pollution sites were significantly higher than that in the shrimp samples collected from the reference site (Table 1), suggesting that oxidative stress was induced by Cd in shrimps. Clearly, both glycine and serine could be used as metabolic markers for Cd exposure in the shrimp *F. chinensis*. The BCAAs, valine, leucine, and isoleucine, were found to be essential for protein synthesis in immune cells for protecting the host from pathogenic invaders (Calder, 2006; De Simone et al., 2013). The decreased level of valine implied the metal pollution-induced disruption of the immune system in the shrimp *F. chinensis*.

Other two BCAAs, namely, isoleucine and leucine, were uniquely depleted in the shrimp samples collected from S6262, implying the more severe disruption of the immune system caused by the higher (~1.6 times, Table 1) Cd accumulation than that in the shrimp samples collected from S7262. Three amino acids, namely, glutamine, asparagine, and lysine, were depleted in the shrimp samples collected from S6262. Amino acids can be oxidized through the tricarboxylic acid cycle (TCA) to provide energy for organisms. In fact, the ATP content was increased in the shrimp samples collected from S6262, which revealed the enhanced oxidation of amino acids and energy storage induced by metal pollution, especially Cd, in shrimp samples.

For the shrimp samples collected from S7262, two organic osmolytes, namely, trimethylamine N-oxide and homarine, were significantly depleted, which suggested the disturbance in osmotic regulation induced by metal pollution in S7262. Amino acids play important roles in energy metabolism, as mentioned above. However, the free amino acids can also be used as osmolytes in osmotic regulation in marine invertebrates to balance the intracellular osmolarity with the environment (Viant et al., 2003). Two amino acids, namely, arginine and proline, were increased in the shrimp samples collected from S7262, which might be used to compensate the decreased levels of trimethylamine N-oxide and homarine related to osmotic regulation. Among these altered amino acids, glycine and proline are crucial for the synthesis of collagen for connective tissue sheaths in the muscle (Cappello et al., 2017). In the posterior adductor muscle of mussels *Mytilus galloprovincialis* caged at the "Augusta-Melilli-Priolo" petrochemical area (Italy), both glycine and proline were elevated, indicating impairment in the muscular structure of the posterior adductor muscle caused by local contaminants such as polycyclic aromatic hydrocarbons (PAHs) and mercury (Hg) (Cappello et al., 2017). In this work, interestingly, both glycine and proline were elevated in the shrimp muscle samples collected from S7262. Although the proline content was not increased, glycine was increased in the shrimp muscle samples collected from S6262. These findings suggested impairment in the muscular structure of shrimps collected from both metal-polluted sites. Inosine is an intermediate involved in the purine nucleotide reactions and hence required for muscle movements (Gómez-Canela et al., 2018). The increased level of inosine suggested the disrupted muscle movement that might be related to the impaired muscular structure in shrimp samples collected from S7262. Malonate is a competitive inhibitor of succinate dehydrogenase that catalyzes the conversion of succinate to fumarate in the TCA cycle and oxidative phosphorylation (Valls-Lacalle et al., 2016). Therefore, the elevated level of malonate might inhibit the conversion of succinate to fumarate in the TCA cycle. Interestingly, the lactate level was elevated, indicating enhanced anaerobiosis in the shrimp samples collected from S7262. Clearly, the metal pollution inhibited aerobic metabolism and enhanced anaerobic metabolism in the shrimp samples collected from S7262, suggesting the disturbed energy metabolism, together with increased phosphocholine.

Overall, the concentrations of Cr and Cu in the shrimp samples collected from S7262 in Laizhou Bay exceeded the national seafood safety standard II (National Standard of PR China, 2005). More severely, the concentration (~11 μg/g wet weight) of As in the shrimp samples collected from S7262 was even higher than the national seafood safety standard III (8.0 μg/g wet weight). These findings suggest us to pay attention to the quality of *F. chinensis,* which is one of the main fishery species in the Bohai Sea. The metabolic responses indicated that the metal pollution induced oxidative and immune stresses and damaged the muscular structure in *F. chinensis* sampled from S6262 and S7262. The metal pollution, especially Cd pollution, enhanced the oxidation of amino acids and energy storage in shrimp samples collected from S6262. The metal pollution in S7262 disturbed the osmotic regulation of the shrimps as revealed by depleted levels of trimethylamine N-oxide and homarine and elevated levels of amino acids, arginine, and proline. Compared with the metabolic profiles of *F. chinensis* collected from S6262, the metal pollution in S7262 disrupted energy metabolism through different pathways by inhibiting aerobic metabolism and enhancing anaerobic metabolism. Furthermore, both glycine and serine could be used as metabolic markers for Cd exposure in *F. chinensis* owing to their altered levels under such condition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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