Baseline

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

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**A B S T R A C T**

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 Liaoqiong Bay, respectively. Our recent studies reported significant Cd and As pollution in the edible shrimp *Cragon annis* 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 shrimp 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...
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 (Na₂HPO₄ and NaH₂PO₄ with 0.5 mM TSP, pH 7.0) in D₂O. 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 ¹H 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 ¹H 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 ¹H 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 HNO₃ 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
Integrated metal contamination = \sum_{i=1}^{m} C_{\text{contaminated}}^{i} - C_{\text{clean}}^{i}

where $C_{\text{contaminated}}^{i}$ is the concentration of the $i$th metal/metalloid in a contaminated shrimp sample, and $C_{\text{clean}}^{i}$ is the reference value for the $i$th metal/metalloid in an uncontaminated shrimp sample. The reference value was determined from the shrimp collected from the relatively clean site S6334. $m$ is the number of all metals/metalloids that a shrimp 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-mine and gold-mining factories in surrounding cities along the Laihou 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 $F. \text{chinensis}$. In addition, the biological effects induced by the metal pollution need to be characterized in the shrimp $F. \text{chinensis}$.

A representative $1^H$ NMR spectrum of muscle tissue extracts of shrimp $F. \text{chinensis}$ 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) acetoadetate, (13) succinate, (14) methionine, (15) histidine, (16) phenylalanine, (17) unknown 1 (2.74 ppm), (18) asparagine, (18) 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.
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 S7262) (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).

The score plots generated by O-PLS-DA could present pairwise
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 Q² 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 samples collected from S7262.

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 aerobic 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 food 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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