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Impact of metal pollution on shrimp *Crangon affinis* by NMR-based metabolomics

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ABSTRACT

Both cadmium and arsenic are the important metal/metalloid pollutants in the Bohai Sea. In this work, we sampled the dominant species, shrimp *Crangon affinis*, from three sites, the Middle of the Bohai Sea (MBS), the Yellow River Estuary (YRE) and the Laizhou Bay (LZB) along the Bohai Sea. The concentrations of metals/metalloids in shrimps *C. affinis* indicated that the YRE site was polluted by Cd and Pb, while the LZB site was contaminated by As. The metabolic differences between shrimps *C. affinis* from the reference site (MBS) and metal-pollution sites (YRE and LZB) were characterized using NMR-based metabolomics. Results indicated that the metal pollutions in YRE and LZB induced disturbances in osmotic regulation and energy metabolism via different metabolic pathways. In addition, a combination of alanine and arginine might be the biomarker of Cd contamination, while BCAAs and tyrosine could be the biomarkers of arsenic contamination in *C. affinis*.

With the rapid development of industry, metal pollution has become a serious environmental problem in the marine and coastal environments in the Bohai Sea. Mu et al. (2009) reported that cadmium (Cd) and arsenic (As) were the dominant metal/metalloid pollutants in the sediments from the Laizhou Bay. In the cultured shellfishes collected from the Laizhou Bay, an early report showed that the samples from more than 50% of the sampling sites were polluted by Cd (Liu et al., 2004). Due to the discharge of industrial effluents along the Yellow River, the Yellow River Estuary has also become a metal-polluted site along the Bohai coast. Since the accumulated metals can induce toxicities in marine organisms, the great concern has been raised over the health risks of metal pollutions to aquatic organisms.

The marine shrimp *Crangon affinis* is the dominant species and widely distributed in the Yellow Sea and the Bohai Sea (Cheng, 2005). *C. affinis* plays a very important role in the food chain since this species is the main bait of fishes. Hence, they can maintain the marine ecosystem health (Cheng, 2005). In addition, shrimp *C. affinis* is consumed as delicious seafood by local residents (Xu et al., 2008). Therefore, it is necessary to investigate the biological effects of metal pollutions to this shrimp species. To our knowledge, however, few studies have been focused on the metal pollution-induced biological effects in shrimp *C. affinis*.

In recent years, the–omics techniques, such as genomics, transcriptomics, proteomics and metabolomics, have been widely used in environmental sciences to elucidate the biological effects induced by environmental stressors to organisms (Ji et al., 2013; Santos et al., 2010; Williams et al., 2009). Metabolomics is a newly established–omics technique that focuses on all the small molecular metabolites (<1000 Da) in organisms (Jones et al., 2008; Viant et al., 2003). A comparative analysis on the metabolome profiles in organisms under environmental stress can provide metabolic responses induced by environmental stressors (Fasulo et al., 2012; Kwon et al., 2012; Cappello et al., 2013). In a previous study, Kwon et al. (2012) successfully applied NMR-based metabolomics to investigate the biological effects of metal pollution in marine mussels (*Mytilus edulis*) sampled from a metal-polluted area (Onsan Bay). Their work confirmed the applicability of NMR-based metabolomics to characterize metal pollution-induced biological effects in organisms.

In this study, the NMR-based metabolomics was used to investigate metabolic responses in shrimp *C. affinis* to metal pollutions. The shrimps *C. affinis* were collected from three sites in the Yellow River Estuary, the Laizhou Bay and the Middle of the Bohai Sea, respectively. Among these sampling sites, the former two sites might be contaminated by metals or metalloids (such as Cd, Pb and As), respectively, and the site in the Middle of the Bohai Sea was a relatively clean and therefore used as the reference. The muscle tissues of individual shrimps *C. affinis* were examined for the metabolic profiles. The aims of this study were to characterize the biological effects in shrimps *C. affinis* exposed to metal pollutions using NMR-based metabolomics.

The shrimps *C. affinis* were collected from the Yellow River Estuary (YRE, 37°45′0″ N, 119°15′0″ E), the Laizhou Bay (LZB, 39°0′0″ N, 120°30′0″ E) and the Middle of the Bohai Sea (MBS, 37°15′0″ N, 119°30′0″ E) along the Bohai Sea in May, 2015 (Fig. 1). Six individual

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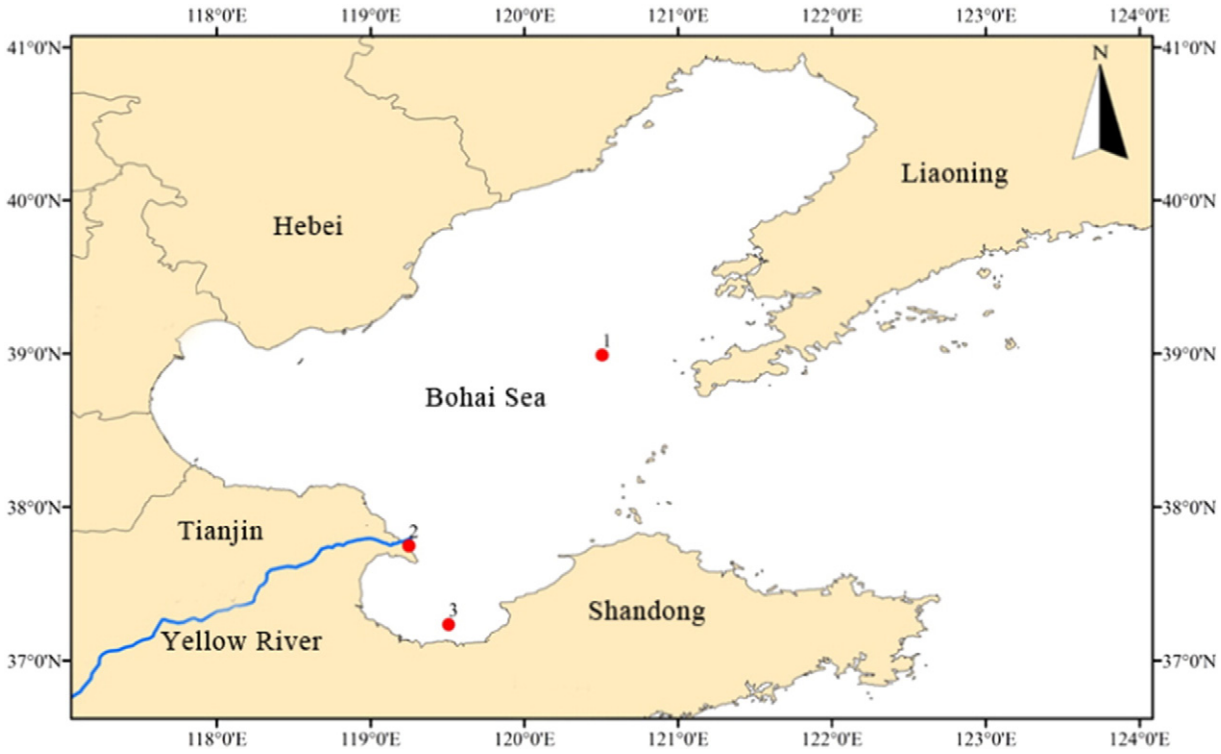

Fig. 1. The map showing the locations of sampling sites in the Bohai Sea, China. 1. The Middle of the Bohai Sea (MBS): 37°15′0″ N, 119°30′0″ E; 2. The Yellow River Estuary (YRE): 37°45′0″ N, 119°15′0″ E; 3. The Laizhou Bay (LZB): 39°0′0″ N, 120°30′0″ E.

shrimps with similar sizes were sampled from each site, and the digestive gland and muscle tissues were immediately dissected and flash frozen in liquid N_2. After transported to our laboratory, these shrimp samples were stored at −80 °C before metabolite extraction and metal analysis. All the practical procedures for shrimp sampling were strictly performed according to the guidelines suggested by Hines et al. (2007) and Vidal-Liñán and Bellas (2013).

Polar metabolites were extracted from the muscle tissues of shrimps ($n = 6$) by a modified extraction protocol using methanol/chloroform (Zhang et al., 2011). Briefly, the muscle tissue (~60 mg) was homogenized using a high throughput homogenizer (Precellys 24, Bertin Technologies, France) and extracted in 4 mL/g of methanol, 0.85 mL/g of water and 2 mL/g of chloroform. The mixture was shaken and centrifuged (5 min, 3000 *g*, at 4 °C), and the supernatant substance was removed. A total of 2 mL/g of chloroform and 2 mL/g of water were added to the supernatant, and the mixture was vortexed and then centrifuged again (10 min, 3000 *g*, 4 °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 °C before NMR measurement. It was subsequently re-suspended 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 at 3000 *g* for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube for NMR analysis.

Extracts of shrimp muscle samples were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 298 K) (Zhang et al., 2011). One-dimensional (1-D) ^{1}H 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, with 128 transients collected into 16 384 data points. Datasets were then zero-filled to 32 768 points, and exponential line-broadenings of 0.3 Hz were applied before Fourier transformation. All ^{1}H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

All one dimensional ^{1}H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab (V7.0, The MathWorks, Natick, MA, USA) (Viant et al., 2003). Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the ^{1}H 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 with a transformation parameter $\lambda = 2.0 \times 10^{-9}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks (Zhang et al., 2011). Data were mean-centered before multivariate data analysis.

The unsupervised pattern recognition method, principal component analysis (PCA) was used to reduce the dimensionality of the data and separate the groups of shrimp samples from MBS, YRE and LZB. 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. Furthermore, the 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 sequentially carried out to uncover and extract the statistically significant metabolite variations related to metal pollutions. The results were visualized in terms of score plots to show the classifications and corresponding loading plots to show the NMR spectral variables contributing to the classifications. The model coefficients were calculated from the coefficients incorporating the weight of the variables in order to enhance interpretability of the model. Then metabolic differences responsible for the classifications between the reference (MBS) and the metal pollution-exposed group (YRE or LZB) could be detected in the coefficient-coded loadings plot generated by using MATLAB (V7.0, the Mathworks Inc., Natwick, USA) with an in-house developed program and was color-coded with absolute value of coefficients (r). A hot color (i.e., red) corresponds to the metabolites with highly positive/negative significances in discriminating between groups, while a cool color (i.e., blue) corresponds to no significance. The correlation coefficient was determined on the basis of the test for the significance of the Pearson's product–moment correlation coefficient. The validation of the model was conducted using 6-fold cross validation and the cross-validation parameter Q^2 was calculated, and an