Cadmium (Cd) has become an important heavy metal contaminant in the sediment and seawater along the Bohai Sea and been of great ecological risk due to its toxic effects to marine organisms. In this work, the toxicological effects caused by environmentally relevant concentrations (10 and 40 μg L⁻¹) of Cd were studied in the gill tissues of Manila clam Ruditapes philippinarum after exposure for 24, 48, and 96 h. Both low (10 μg L⁻¹) and high (40 μg L⁻¹) doses of Cd caused the disturbances in energy metabolism and osmotic regulation and neurotoxicity based on the metabolic biomarkers such as succinate, alanine, branched chain amino acids, betaine, hypotaurine, and glutamate in clam gills after 24 h of exposure. However, the recovery of toxicological effects of Cd after exposure for 96 h was obviously observed in clam to Cd exposures. Overall, these results indicated that NMR-based metabolomics was applicable to elucidate the toxicological effects of heavy metal contaminants in the marine bioindicator.

Keywords: Biomarker; Cadmium; Manila clam; Metabolomics; Toxicological effect

Received: April 19, 2011; revised: July 18, 2011; accepted: July 27, 2011
DOI: 10.1002/clen.201100208

1 Introduction

Cadmium (Cd) has become a serious pollutant in the Bohai marine and coastal ecosystems as a result of natural and anthropogenic activities. In some heavily polluted estuaries or harbors, the concentration of Cd has been as high as 50 μg L⁻¹ with the discharge of industrial wastewaters [1]. In the Bohai marine and coastal ecosystems, Cd has become one of the most serious contaminants as well due to the industrial discharge from numerous metal smelteries [2]. Cd can be accumulated by organisms and transferred to the human body by food chain [1, 3–5]. It is known that chronic Cd poisoning could induce the itai-itai disease and carcinogenesis [6, 7]. To assess the impact of Cd, the toxicological effects should be characterized in resident biota using effective toxicological approaches [8–10].

Some traditional toxicological techniques have been frequently used to test the toxicities on the basis of measurements of specific biochemical indices, such as the acetylcholinesterase (AChE) activity to examine neurotoxicity or the antioxidant enzymatic activities to illustrate the oxidative stresses induced by toxicants [8, 9, 11, 12]. Due to the rapid developments in system biology, the new toxicological approaches including proteomics, transcriptomics, and metabolomics have been proved powerful to illustrate the deleterious effects of toxicants in organisms [13–15].

Metabolomics, one of the techniques of system biology, is defined as “systematic study of the unique chemical fingerprints (metabolic biomarkers) that cellular processes leave behind” [16]. It has been widely applied in multiple areas such as drug toxicity, ecotoxicology, and plant sciences [17–22]. The nuclear magnetic resonance (NMR) spectroscopy-based metabolomics has been successfully detected the responses of metabolites with low molecular weight (<1000 Da) to toxic insults has been successfully employed in both terrestrial vertebrate and invertebrate relevant to ecotoxicology [23–25]. ¹H-NMR spectroscopy is uniquely suitable for the detection of numerous endogenous metabolites in the biological samples (e.g., biological fluids, tissue extracts, and intact tissues) since all the metabolite molecules practically contain proton (s). Although NMR-based metabolomics has been successfully applied in multiple areas, very few studies on the toxicology of marine contaminants in invertebrates has been reported [26–28].

Clam Ruditapes philippinarum is an economic species of fishery in China and used as the preferred biomonitor in the “Mussel Watch Programs”. Since clam is widely distributed along the Bohai coast and this species possesses long life history, exceptional tolerance to environmental factors (e.g., salinity and temperature), and can accumulate high amounts of contaminants, it has been also employed in marine environmental toxicology as a bioindicator [29–32].

Since gill tissue in mollusks can accumulate considerable amounts of contaminants [33], it is frequently applied for the discovery of toxicological effects of contaminants. In the present study, gill tissue extracts from R. philippinarum were extracted and analyzed to characterize metabolic biomarkers caused by two environmentally relevant doses of Cd using ¹H-NMR-based metabolomics. The aim of this study was to detect metabolic responses to illustrate the toxicological effects induced by Cd in adult Manila clams after three (24, 48, and 96 h) exposure times.

Abbreviations: AChE, acetylcholinesterase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FDR, false discovery rate; NMR, nuclear magnetic resonance; PCA, principal component; PPR, principal component analysis; PR, pattern recognition

Correspondence: Dr. H. Wu, Key Laboratory of Coastal Zone Environment Processes, CAS; Shandong Provincial Key Laboratory of Coastal Zone Environment Processes, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, P. R. China.

E-mail: hfwu@yic.ac.cn

© 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

www.clean-journal.com
2 Materials and methods

2.1 Clam exposure

Adult Manila clams R. philippinarum (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from a local culturing farm in Yantai, China. Animals were allowed to acclimatize in aerated seawater (25°C, 32 psu, collected from pristine environment) in the laboratory for 10 days and fed with the Chlorella vulgaris Beij daily. After the acclimatization period, the clams were randomly divided into three flat-bottomed rectangular tanks with 20L capacity and each tank contained 20 individual clams.

For the exposure experiment, clams were exposed to Cd at two environmentally relevant concentrations of 10 and 40 µg·L⁻¹. These concentrations of Cd can be found in the polluted sites along the Bohai coast, China. Each tank contained 20 individual clams. After exposure for 24, 48, and 96 h, respectively. After exposure for 24, 48, or 96 h, the clams from each tank were randomly removed and dissected. The gill tissue samples were flash-frozen in liquid nitrogen immediately and stored at −80°C prior to metabolite extraction.

2.2 Metabolite extraction

Polar metabolites were extracted from the gill tissues using a modified methanol/chloroform solvent system as described previously [34–38]. Briefly, the gill tissue (~100 mg) was homogenized in 4 mL·g⁻¹ (solvent volume/tissue mass, the same to below) of methanol and 0.85 mL·g⁻¹ of water using a high throughput homogenizer, Precellys 24 (Bertin, France). The mixture was transferred to a glass vial. A total of 2 mL·g⁻¹ of chloroform and 2 mL·g⁻¹ of water was added to the mixture, and the mixture was vortexed and centrifuged again (10 min, 2000 × g, 4°C). The methanol/water layer with the polar metabolites from clam gill tissue was removed and dried in a centrifugal concentrator and then stored at −80°C. The metabolite extracts was subsequently resolved in 600 µL of 150 mM phosphate buffer (Na₂HPO₄ and NaH₂PO₄, pH 7.0) with 0.5 mM sodium 3-trimethylsilyl-2,2,3,3-d₄-propionate (TSP) as chemical shift standard in D₂O. The mixture was vortexed and then centrifuged at 2500 × g for 5 min at 4°C. The supernatant (550 µL) was then pipetted into a 5 mm NMR tube before NMR measurement.

2.3 NMR spectroscopy

The tissue extracts were analyzed using a Bruker AV 500 NMR spectrometer operated at 500.18 MHz at 298 K as described previously [37–39]. One-dimensional (1D) ¹H-NMR spectra were obtained using a 11.9 µs pulse, 6009.6 Hz spectral width, mixing time 0.1 and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, 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 tabulated chemical shifts [40, 41] and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada).

2.4 Spectral pre-processing and multivariate data analysis

All the NMR spectra were converted to a format for pattern recognition (PR) analysis using custom-written ProMetab software based on the Matlab software package as described previously (version 7.0; The MathWorks, Natick, MA) [28, 37–39, 42]. Each 1H-NMR spectrum was segmented into 0.01 ppm bins between 0.2 and 10.0 ppm with bins from 4.60 to 5.20 ppm (the residual water peak) excluded. The area of each segment was calculated and normalized using the total integrated spectral area of the spectrum. All the NMR spectra were log transformed (with transformation parameter, λ = 1 × 10⁻⁶) to stabilize the variance across the spectral bins and to enhance the weightings of the less intense peaks [42, 43]. The data sets were mean-centered before principal components analysis (PCA) was performed using PLS Toolbox software (version 4.0, Eigenvector Research, Manson, WA).

PCA is an exploratory unsupervised PR technique which is blind to the status of each sample, and serves to reduce the dimensionality of the data and summarize the similarities and differences between multiple NMR spectral sets [44]. The algorithm of this PR method calculates the highest amount of correlated variation along principal component (PC1), with subsequent PCs containing correspondingly smaller amounts of variance. One-way analysis of variance (ANOVA) with Tukey’s test was performed on the PC scores of various groups of samples to test the significance of separations between the control and Cd-exposed groups. For each model built, the loading vector for the PC could be examined to identify the metabolites which contributed to the clusters. SAM software [45] was then used to find significant metabolic differences among Cd-exposed groups with appropriate false discovery rate (FDR) cutoffs. Bins that changed significantly (at FDR < 0.01) were subsequently identified using Chenomx software and isolated. For the identification of significant metabolites, one-way ANOVA was conducted on the ratio of significantly changed bin (at FDR < 0.01) area (peak intensity) of metabolites to the total spectral area [22, 46]. These significant metabolites were contributive for the separation between control and Cd-treated samples and hence were considered metabolic biomarkers induced by Cd exposures. A p-value of 0.05 was considered significant for the multiple comparisons on the metabolites between control and exposed samples.

3 Results

3.1 ¹H-NMR spectroscopy of clam gill tissue extracts

Figure 1 shows a typical 1D ¹H-NMR spectrum of gill tissue extracts from a control Manila clam (Fig. 1). After log transformation, the relative NMR peak intensities of less abundant metabolites such as homarine were obviously enhanced after glog-transformation (data not shown). Several different classes of metabolites were identified by Chenomx software, including amino acids (e.g., aspartate, glutamate, and glycine), energy storage compounds (glucose and glycogen), organic osmolytes (betaine, homarine, etc.), and intermediates in citric acid cycle (e.g., succinate).

3.2 Principal components analysis on the ¹H-NMR spectra of gill tissue extracts

PCA was performed on the NMR spectral data of gill tissue extracts after 24, 48, and 96 h exposures with Cd. After 24 h of exposure, the
gill samples from control (inverted red triangles) and high dose (40 µg L \(^{-1}\)) of Cd-exposed groups were significantly separated along PC2 (\(p < 0.05\)), while the low dose (10 µg L \(^{-1}\), green cycles) of Cd-exposed group was clustered upon both negative PC1 and PC2 axes (Fig. 2a). The scores plot (PC1 vs. PC4) showed significant (\(p < 0.05\)) separation between 10 µg L \(^{-1}\) Cd-exposed and control samples along PC1 axis after exposure for 48 h as well as the significant (\(p < 0.05\)) separation between 40 µg L \(^{-1}\) Cd-exposed and control samples along PC4 axis (Fig. 3a). For the exposure after 96 h, there was no significant (\(p > 0.05\)) separation between Cd-exposed and control groups. However, the metabolic differences between control and high dose (40 µg L \(^{-1}\)) of Cd-exposed group along PC2 axis approached to statistical significance (ANOVA, \(p = 0.069\); Fig. 4a).

The plots (Figs. 2b and c, 3b) of PC loading were originally used to define the NMR spectral bins (peaks) of metabolites which might be significant for the separations between Cd-dosed and control groups. Then, one-way ANOVA with a 5% significance level (at FDR < 0.01) was applied to the NMR spectral bins that presented a possible contribution to the separation, facilitating the identification of metabolic changes.

Basically, the levels of alanine, succinate, hypotaurine, and homarine were significantly down-regulated in the gill tissues from the low (10 µg L \(^{-1}\)) Cd-treated group after 24 h of exposure, while the level of branched chain amino acids, aspartate and betaine were up-regulated significantly (Tab. 1 and Fig. 2b). For the separation between high dose (40 µg L \(^{-1}\)) of Cd-exposed and control groups, the metabolic differences were very similar to that between 10 µg L \(^{-1}\) Cd-treated and control groups with the exception of significantly increased glutamate in 40 µg L \(^{-1}\) Cd-treated samples (Tab. 1 and Fig. 2c). After 48 h of Cd exposure, the significant metabolic responses in gill tissues induced by 10 µg L \(^{-1}\) Cd were the decreased succinate and increased glutamate and aspartate (Tab. 1 and Fig. 3b). The significant (\(p < 0.05\)) metabolic differences
resulted in high abundances of branched chain amino acids and aspartate in high dose (40 μg L⁻¹/C₀) of Cd-exposed samples as well as the depletion of acetate and succinate (Tab. 1 and Fig. 3c). For the exposure of 96 h, the metabolic responses induced by 40 μg L⁻¹/C₀ Cd included the depletion of succinate and the elevation of aspartate.

4 Discussion

In the original NMR spectrum (Fig. 1), malonate was found to be one dominant metabolite in gill tissues. Malonate is a three-carbon dicarboxylic acid that can be found in diverse organism tissues including in the soybean tissues, rat brain, earthworm, fish liver, and mussel [46–49]. Organic osmolytes such as betaine, homarine, hypotaurine, dimethylamine, alanine (also involved in energy metabolism), and taurine are small organic compounds that play key roles in the osmotic regulation in marine invertebrates via various metabolic pathways and were therefore found at relatively higher levels in clams [50]. These organic osmolytes can be accumulated or released depending on the salinity of seawater.

After PCA analysis, the PC score plots showed clear separations between the control and two Cd-exposed groups after either 24 or 48 h of exposure (Figs. 2a and 3a), which exhibited the metabolic responses induced by these two doses of Cd in clam gill samples. However, the samples of low (10 μg L⁻¹/C₀) concentration of Cd-exposed group were clustered to the control group after exposure for 96 h. It seemed that the metabolic profiles of the 10 μg L⁻¹/C₀ Cd-exposed group returned to the control level, which meant the recovery of toxicological effects induced by 10 μg L⁻¹ Cd post 96 h. It could be accounted for the biological adaptation of clam to Cd exposures after 96 h.

After 24 h of exposure of Cd, the alteration in osmolytes including the elevation of betaine and reduction of homarine and hypotaurine in both low (10 μg L⁻¹) dose of Cd-treated samples exhibited the disturbances in osmotic regulation in clam gills. Viant et al. [41] have reported that red abalone *Haliotis rufescens* accumulated high amounts of amino acids to balance the intracellular osmolarity with environment [41]. The increase of branched chain amino acids in
clam gill tissues indicated disturbances in osmolarity caused by the exposure to Cd. Alanine and succinate are the end-products of glucose breakdown anaerobically in invertebrate [51, 52]. However, both succinate and alanine were down-regulated in this work after exposure of Cu for 24 h, which implied that the alterations in succinate and alanine were independently involved in other metabolic pathways such as the osmotic regulation of alanine but in the anaerobic metabolism. The phenomenon was discovered in mercury-exposed clams as well in our previous work [35]. As a matter of fact, succinate is a key intermediate in the tricarboxylic acid cycle that is related to energy metabolism, therefore, the decrease of succinate in both 10 and 40 μg L^{-1} Cd-exposed groups meant the possible perturbations in energy metabolism induced by Cd after 24 h of exposure. In the 40 μg L^{-1} Cd-exposed samples, the elevation of glutamate was differentially observed and it was suggested as a biomarker of neurotoxicity [53–55].

Compared to the metabolic changes induced by Cd exposures after 24 h, the altered osmoieties including the elevated betaine and depleted hypotaurine and homarine recovered to the control level after either 48 or 96 h of exposure. It indicated the recovery of disturbances in osmotic regulations. However, the decreased acetate was uniquely detected in the high dose (40 μg L^{-1}) Cd-treated clams post 48 h. Acetate is an end-product of anaerobic metabolism that can be used as a biomarker of anoxic conditions with elevated concentrations in the biological tissues [56, 57]. In this work, the significantly decreased acetate in clam gill tissues after exposed to Cd for 48 h indicated the reduced anaerobic metabolism in gills. Although the samples from the 40 μg L^{-1} Cd-exposed group were not significantly (p = 0.069 > 0.05) separated from the control group, a couple of metabolic responses including the elevated aspartate and depleted succinate were detected in Cd-exposed clam gill extracts after exposure for 96 h. However, other detectable metabolic biomarkers such as elevated branched amino acids recovered to the control levels, which indicated the recovery of metabolic changes caused by both 10 and 40 μg L^{-1} Cd after exposure for 96 h.

### 5 Conclusions

This work characterized the toxicological effects in the gill tissues of Manila clam R. philippinarum caused by two doses (10 and 40 μg L^{-1}) of Cd during a 96-h exposure course. The dose- and time-dependent toxicological effects of Cd exposures included the disturbances in energy metabolisms and osmotic regulations and potential neurotoxicity based on the corresponding metabolic biomarkers such as succinate, branched chain amino acids, aspartate, betaine, hypotaurine glutamate, etc. Our findings indicated that NMR-based metabolomics was applicable to elucidate the toxicological effects of heavy metal contaminants in the marine bioindicator.

### Acknowledgments

We thank Dr. Mark Viant (School of Bioscience, The University of Birmingham) for use of the software ProMetab. This research was supported by The 100 Talents Program of the Chinese Academy of Sciences, NSFC (41106102), Technology Development Program Projects of Shandong Province (2008GG2000506 and 2008GG2NS0700) and SDSFC (ZR2009CZ008) and in part by the CAS/SAFEA International Partnership Program for Creative Research Teams “Representative environmental processes and resources effects in coastal”.

The authors have declared no conflict of interest.

### References


