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Research Article

Toxicological Effects Induced by Cadmium in Gills of Manila Clam *Ruditapes philippinarum* Using NMR-Based Metabolomics

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 $\mu g\,L^{-1}$) of Cd were studied in the gill tissues of Manila clam *Ruditapes philippinarum* after exposure for 24, 48, and 96 h. Both low (10 $\mu g\,L^{-1}$) and high (40 $\mu g\,L^{-1}$) 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

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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 $\mu g\,L^{-1}$ 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].

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

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 to detect 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]. ^{1}H-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 molusks 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 ^{1}H-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.

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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 20 L 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 $\mu g\,L^{-1}$. These concentrations of Cd can be found in the polluted sites along the Bohai coast [2], and, therefore, were used for the exposures to clam *R. philippinarum* in this work. The calms cultured in the normal filtered seawater were used as control group. The animals ($n = 5$) were randomly sampled 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, $2000 \times g$, 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 (Na_2HPO_4 and NaH_2PO_4, pH 7.0) with 0.5 mM sodium 3-trimethylsilyl-2,2,3,3-*d*4-propionate (TSP) as chemical shift standard in D_2O. The mixture was vortexed and then centrifuged at $2500 \times g$ 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) ^{1}H-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 ^{1}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 [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 ^{1}H-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, $\lambda = 1 \times 10^{-8}$) 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 ^{1}H-NMR spectroscopy of clam gill tissue extracts

Figure 1 shows a typical 1D ^{1}H-NMR spectrum of gill tissue extracts from a control Manila clam (Fig. 1). After glog 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 ^{1}H-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

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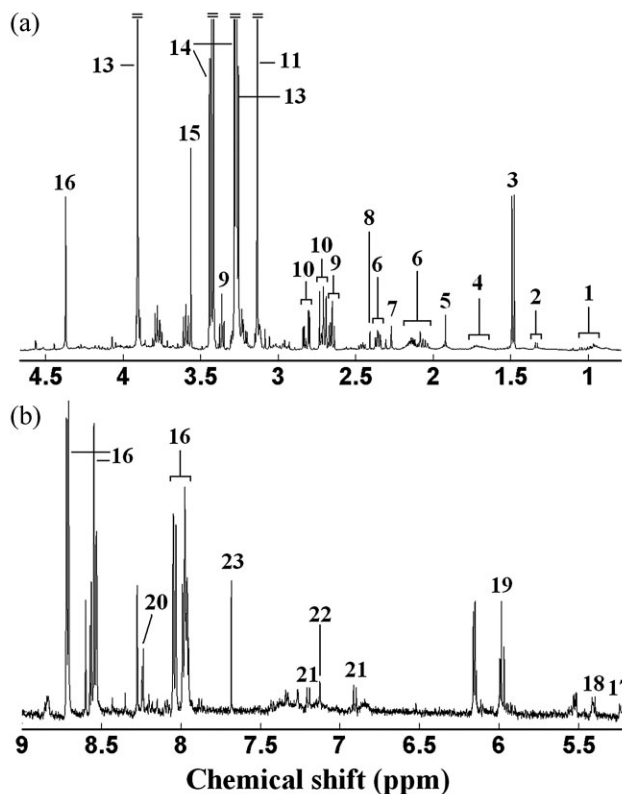

Figure 1. A representative 1D 500 MHz ^{1}H-NMR spectrum of gill tissue extracts from a control clam (a) vertical expansion of the aromatic region (b). Keys: (1) Branched chain amino acids: Isoleucine, leucine, and valine, (2) lactate, (3) alanine, (4) arginine, (5) acetate, (6) glutamate, (7) aceto-acetate, (8) succinate, (9) hypotaurine, (10) aspartate, (11) malonate, (12) acetylcholine, (13) betaine, (14) taurine, (15) glycine, (16) homarine (17) glucose, (18) glycogen, (19) unknown 1 (5.95 ppm), (20) adenosine triphosphate (ATP)/adenosine diphosphate (ADP), (21) tyrosine, (22) histidine, and (23) unknown 2 (7.68 ppm).

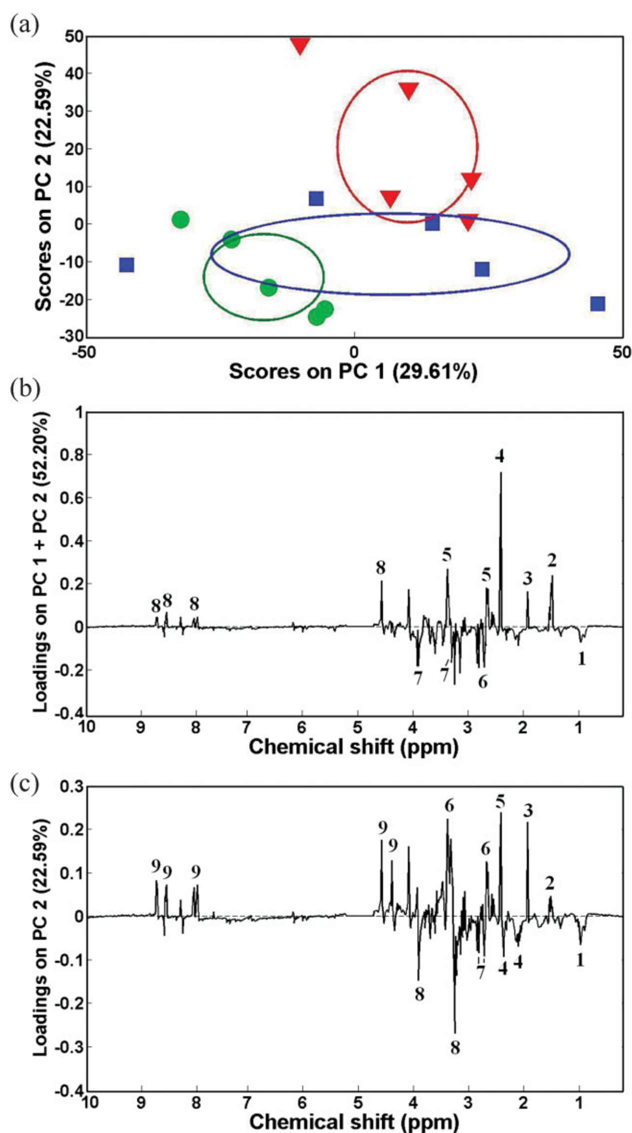

Figure 2. PCA showing (a) separations (PC1 vs. PC2) between control (▼), 10 (●), and 40 $\mu g\,L^{-1}$ (■) Cd-exposed clam samples after exposure for 24 h, and corresponding PC1 (b) and PC2 (c) loadings plots. Ellipses represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Keys in (b): (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) alanine, (3) acetate, (4) succinate, (5) hypotaurine, (6) aspartate, (7) betaine, and (8) homarine. Keys in (c): (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) alanine, (3) acetate, (4) glutamate, (5) succinate, (6) hypotaurine, (7) aspartate, (8) betaine, and (9) homarine.

gill samples from control (inverted red triangles) and high dose (40 $\mu g\,L^{-1}$, blue squares) of Cd-exposed groups were significantly separated along PC2 ($p < 0.05$), while the low dose (10 $\mu g\,L^{-1}$, 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 $\mu g\,L^{-1}$ Cd-exposed and control samples along PC1 axis after exposure for 48 h as well as the significant ($p < 0.05$) separation between 40 $\mu g\,L^{-1}$ 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 $\mu g\,L^{-1}$) 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 $\mu g\,L^{-1}$) 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 $\mu g\,L^{-1}$) of Cd-exposed and control groups, the metabolic differences were very similar to that between 10 $\mu g\,L^{-1}$ Cd-treated and control groups with the exception of significantly increased glutamate in 40 $\mu g\,L^{-1}$ Cd-treated samples (Tab. 1 and Fig. 2c). After 48 h of Cd exposure, the significant metabolic responses in gill tissues induced by 10 $\mu g\,L^{-1}$ Cd were the decreased succinate and increased glutamate and aspartate (Tab. 1 and Fig. 3b). The significant ($p < 0.05$) metabolic differences

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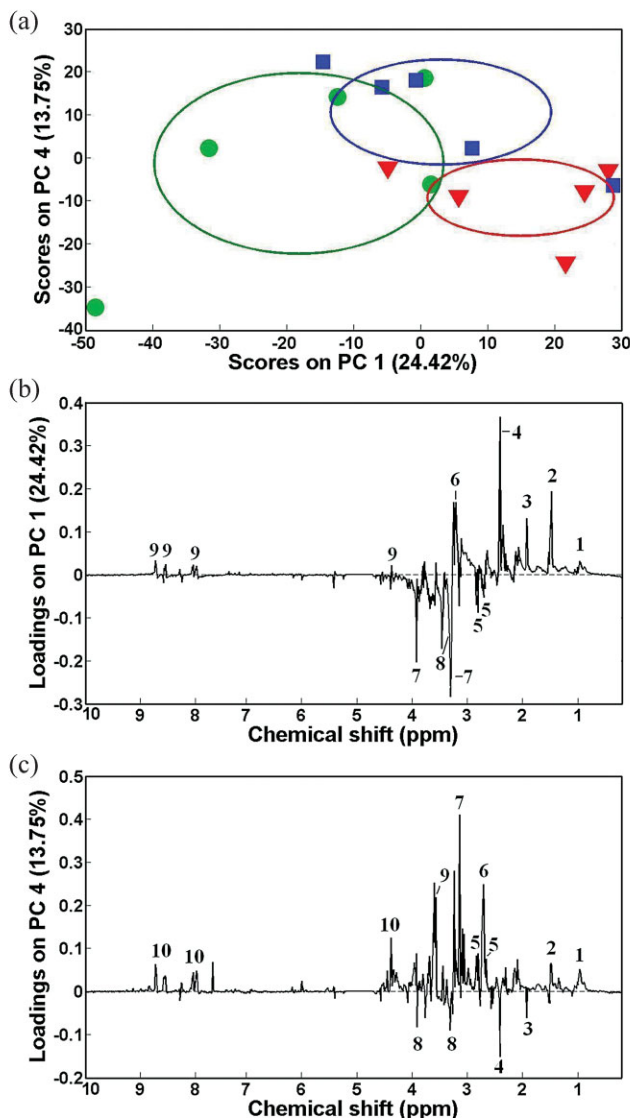

Figure 3. PCA showing (a) separations (PC1 vs. PC4) between control (▼), 10 (●), and 40 $\mu g\,L^{-1}$ (■) Cd-exposed clam samples after exposure for 48 h, and corresponding PC1 (b) and PC4 (c) loadings plots. Ellipses represented mean ± SD of PC scores along both PC1 and PC4 axes for each group. Keys in (b): (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) alanine, (3) acetate, (4) succinate, (5) aspartate, (6) phosphocholine, (7) betaine, and (8) taurine. Keys in (c): (1) Branched chain amino acids, (2) alanine, (3) acetate, (4) succinate, (5) aspartate, (6) dimethylamine, (7) malonate, (8) betaine, (9) glycine, and (10) homarine.

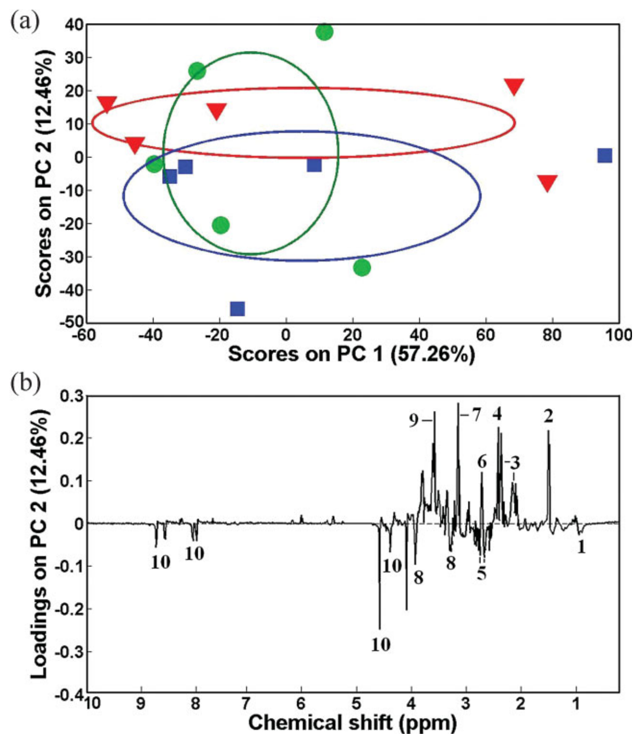

Figure 4. PCA showing (a) separations (PC1 vs. PC2) between control (▼), 10 (●), and 40 $\mu g\,L^{-1}$ (■) Cd-exposed clam samples after exposure for 96 h, and corresponding PC2 (b) loadings plot. Ellipses represented mean ± SD of PC scores along both PC1 and PC2 axes for each group. Keys in (b): (1) Branched chain amino acids: isoleucine, leucine, and valine, (2) alanine, (3) glutamate, (4) succinate, (5) aspartate, (6) dimethylamine, (7) malonate, (8) betaine, (9) glycine, and (10) homarine.

resulted in high abundances of branched chain amino acids and aspartate in high dose (40 $\mu g\,L^{-1}$) 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 $\mu g\,L^{-1}$ 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 $\mu g\,L^{-1}$) 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 $\mu g\,L^{-1}$ Cd-exposed group returned to the control level, which meant the recovery of toxicological effects induced by 10 $\mu g\,L^{-1}$ 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 $\mu g\,L^{-1}$) 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

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Table 1. Significantly up- or down-regulated metabolites ($p < 0.05$[a)]) in gill tissues of Cd-exposed clams after exposures for 24, 48, and 96 h.

Dose	10 $\mu g\ Cd^{2+}\ L^{-1}$ seawater		40 $\mu g\ Cd^{2+}\ L^{-1}$ seawater	
Exposure time	Up-regulated metabolites	Down-regulated metabolites	Up-regulated metabolites	Down-regulated metabolites
24 h	Branched chain amino acids: aspartate and betaine	Alanine, succinate, hypotaurine, and homarine	Branched chain amino acids: glutamate, aspartate, and betaine	Alanine, succinate, hypotaurine, and homarine
48 h	Branched chain amino acids	Succinate	Branched chain amino acids: aspartate	Acetate and succinate
96 h	ND	ND	Aspartate	succinate

ND, not detected.
a) *p*-Values determined using one-way ANOVA on the bin areas from the representative peak of corresponding metabolite.

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 $\mu g\ L^{-1}$ Cd-exposed groups meant the possible perturbations in energy metabolism induced by Cd after 24 h of exposure. In the 40 $\mu g\ L^{-1}$ 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 osmolytes 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 $\mu g\ L^{-1}$) 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 $\mu g\ L^{-1}$ 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 $\mu g\ L^{-1}$ 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 $\mu g\ L^{-1}$) 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.

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References

[1] R. Chester, *Marine Geochemistry*, Allen & Unwin, London **1990**, p. 698.

[2] X. Zhang, Investigation of Pollution of Pb, Cd, Hg, As in Sea Water and Deposit of Bohai Sea Area, *Heilongjiang Environ. J.* **2001**, *25*, 87–890.

[3] A. Sahmurova, H. Türkmenler, E. E. Özbaş, Biosorption Kinetics and Isotherm Studies of Cd(II) by Dried *Enteromorpha compressa* Macroalgae Cells from Aqueous Solutions, *Clean – Soil Air Water* **2010**, *38*, 936–941.

[4] F. M. Morsy, S. H. A. Hassan, M. Koutb, Biosorption of Cd(II) and Zn(II) by *Nostoc commune*: Isotherm and Kinetics Studies, *Clean – Soil Air Water* **2011**, *39*, 680–687.

[5] T. Tay, M. Candan, M. Erdem, Y. Cimen, H. Türk, Biosorption of Cadmium Ions from Aqueous Solution Onto Non-Living Lichen *Ramalina fraxinea* Biomass, *Clean – Soil Air Water* **2009**, *37*, 249–255.

[6] A. Takaki, S. Jimi, M. Segawa, S. Hisano, S. Takebayashi, H. Iwasaki, Long-Term Cadmium Exposure Accelerates Age-Related Mitochondrial Changes in Renal Epithelial Cells, *Toxicology* **2004**, *203*, 145–154.

[7] G. Bertin, D. Averbeck, Cadmium: Cellular Effects, Modifications of Biomolecules, Modulation of DNA Repair and Genotoxic Consequences (a Review), *Biochimie* **2006**, *88*, 1549–1559.

[8] T. S. Galloway, R. C. Sanger, K. L. Smith, G. Fillmann, J. W. Readman, T. E. Ford, M. H. Depledge, Rapid Assessment of Marine Pollution Using Multiple Biomarkers and Chemical Immunoassays, *Environ. Sci. Technol.* **2002**, *36*, 2219–2226.

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[9] Q. Y. Huang, C. W. Fang, H. Q. Huang, Alteration of Heart Tissue Protein Profiles in Acute Cadmium-Treated Scallops *Patinopecten yessoensis*, *Arch. Environ. Contam. Toxicol.* **2010**, *60*, 90–98.

[10] S. K. Sheir, R. D. Handy, Tissue Injury and Cellular Immune Responses to Cadmium Chloride Exposure in the Common Mussel *Mytilus edulis*: Modulation by Lipopolysaccharide, *Arch. Environ. Contam. Toxicol.* **2010**, *59*, 602–613.

[11] V. Matozzo, A. Tomei, M. Marin, Acetylcholinesterase as a Biomarker of Exposure to Neurotoxic Compounds in the Clam *Tapes philippinarum* from the Lagoon of Venice, *Mar. Pollut. Bull.* **2005**, *50*, 1686–1693.

[12] A. Elbaz, Y. Y. Wei, Q. Meng, Q. Zheng, Z. M. Yang, Mercury-Induced Oxidative Stress and Impact on Antioxidant Enzymes in *Chlamydomonas reinhardtii*, *Ecotoxicology* **2010**, *19*, 1285–1293.

[13] C. Saavedra, E. Bachere, Bivalve Genomics, *Aquaculture* **2006**, *256*, 1–14.

[14] F. Dondero, A. Dagnino, H. Jonsson, F. Capri, L. Gastaldi, A. Viarengo, Assessing the Occurrence of a Stress Syndrome in Mussels (*Mytilus edulis*) Using a Combined Biomarker/Gene Expression Approach, *Aquat. Toxicol.* **2006**, *78*, 13–24.

[15] F. Dondero, L. Piacentini, F. Marsano, M. Rebelo, L. Vergani, P. Venier, A. Viarengo, Gene Transcription Profiling in Pollutant Exposed Mussels (*Mytilus* spp.) Using a New Low-Density Oligonucleotide Microarray, *Gene* **2006**, *376*, 24–36.

[16] B. Daviss, Growing Pains for Metabolomics, *Scientist* **2005**, *19*, 25–28.

[17] H. Wu, X. Zhang, X. Li, Y. Wu, F. Pei, Acute Biochemical Effects of $La(NO_3)_3$ on Liver and Kidney Tissues by Magic-Angle Spinning ^{1}H-Nuclear Magnetic Resonance Spectroscopy and Pattern Recognition, *Anal. Biochem.* **2005**, *339*, 242–248.

[18] C. L. Gavaghan, I. D. Wilson, J. K. Nicholson, Physiological Variation in Metabolic Phenotyping and Functional Genomic Studies: Use of Orthogonal Signal Correction and PLS-DA, *FEBS Lett.* **2002**, *530*, 191–196.

[19] J. T. Brindle, H. Antti, E. Holmes, G. Tranter, J. K. Nicholson, H. W. L. Bethell, S. Clarke, et al., Rapid and Noninvasive Diagnosis of the Presence and Severity of Coronary Heart Disease Using ^{1}H-NMR-Based Metabonomics, *Nat. Med.* **2002**, *8*, 1439–1444.

[20] J. G. Bundy, D. J. Spurgeon, C. Svendsen, P. K. Hankard, J. M. Weeks, D. Osborn, J. C. Lindon, J. K. Nicholson, Environmental Metabonomics: Applying Combination Biomarker Analysis in Earthworms at a Metal Contaminated Site, *Ecotoxicology* **2004**, *13*, 797–806.

[21] M. R. Viant, C. A. Pincetich, D. E. Hinton, R. S. Tjeerdema, Toxic Actions of Dinoseb in Medaka (*Oryzias latipes*) Embryos as Determined by *in Vivo* ^{31}P-NMR, HPLC-UV and ^{1}H-NMR Metabolomics, *Aquat. Toxicol.* **2006**, *76*, 329–342.

[22] I. Katsiadaki, T. D. Williams, J. S. Ball, T. P. Bean, M. B. Sanders, H. Wu, E. M. Santos, et al., Hepatic Transcriptomic and Metabolomic Responses in the Stickleback (*Gasterosteus aculeatus*) Exposed to Ethinyl-Estradiol, *Aquat. Toxicol.* **2009**, *43*, 6341–6348.

[23] J. G. Bundy, E. M. Lenz, N. J. Bailey, C. L. Gavaghan, C. Svendsen, D. Spurgeon, P. K. Hankard, et al., Metabonomic Assessment of Toxicity of 4-Fluoroaniline, 3,5-Difluoroaniline and 2-Fluoro-4-methylaniline to the Earthworm *Eisenia veneta* (Rosa): Identification of New Endogenous Biomarkers, *Environ. Toxicol. Chem.* **2002**, *21*, 1966–1972.

[24] J. L. Griffin, L. A. Walker, S. Garrod, E. Holmes, R. F. Shore, J. K. Nicholson, NMR Spectroscopy Based Metabonomic Studies on the Comparative Biochemistry of the Kidney and Urine of the Bank Vole (*Clethrionomys glareolus*), Wood Mouse (*Apodemus sylvaticus*), White Toothed Shrew (*Crocidura suaveolens*) and the Laboratory Rat, *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* **2000**, *127*, 357–367.

[25] O. Fiehn, Metabolomics: The Link between Genotypes and Phenotypes, *Plant Mol. Biol.* **2002**, *48*, 155–171.

[26] O. A. H. Jones, F. Dondero, A. Viarengo, J. L. Griffin, Metabolic Profiling of *Mytilus Galloprovincialis* and Its Potential Applications for Pollution Assessment, *Mar. Ecol. Prog. Ser.* **2008**, *369*, 169–179.

[27] W. Tuffnail, G. A. Mills, P. Cary, R. Greenwood, An Environmental ^{1}H-NMR Metabolomic Study of the Exposure of the Marine Mussel *Mytilus edulis* to Atrazine, Lindane, Hypoxia and Starvation, *Metabolomics* **2009**, *5*, 33–43.

[28] H. Wu, W.-X. Wang, NMR-Based Metabolomic Studies on the Toxicological Effects of Cadmium and Copper on Green Mussels *Perna viridis*, *Aquat. Toxicol.* **2010**, *100*, 339–345.

[29] K.-I. Park, C. Paillard, P. L. Chevalier, K.-S. Choi, Report on the Occurrence of Brown Ring Disease (BRD) in Manila Clam, *Ruditapes philippinarum*, on the West Coast of Korea, *Aquaculture* **2006**, *255*, 610–613.

[30] K.-I. Park, H. Tsutsumi, J.-S. Hong, K.-S. Choi, Pathology Survey of the Short-Neck Clam *Ruditapes philippinarum* Occurring on Sandy Tidal Flats along the Coast of Ariake Bay, Kyushu, Japan, *J. Invert. Pathol.* **2008**, *99*, 212–219.

[31] J. Ji, H. J. Choi, I.-Y. Ahn, Evaluation of Manila Clam *Ruditapes philippinarum* as a Sentinel Species for Metal Pollution Monitoring in Estuarine Tidal Flats of Korea: Effects of Size, Sex, and Spawning on Baseline Accumulation, *Mar. Pollut. Bull.* **2006**, *52*, 447–468.

[32] N. Liu, L. Pan, J. Miao, C. Xu, L. Zhang, Molecular Cloning and Sequence Analysis and the Response of an Aryl Hydrocarbon Receptor Homologue Gene in the Clam *Ruditapes philippinarum* Exposed to Benzo(*a*)pyrene, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2010**, *152*, 279–287.

[33] I. Panfoli, B. Burlando, A. Viarengo, Effects of Heavy Metals on Phospholipase C in Gill and Digestive Gland of the Marine Mussel *Mytilus galloprovincialis* Lam, *Comp. Biochem. Physiol., Part B: Toxicol. Pharmacol.* **2000**, *127*, 391–397.

[34] E. G. Bligh, W. J. Dyer, A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

[35] X. Liu, L. Zhang, L. You, J. Yu, J. Zhao, L. Li, Q. Wang, et al., Differential Toxicological Effects Induced by Mercury in Gills from Three Pedigrees of Manila Clam *Ruditapes philippinarum* by NMR-Based Metabolomics, *Ecotoxicology* **2011**, *20*, 177–186.

[36] H. Wu, A. D. Southam, A. Hines, M. R. Viant, High Throughput Tissue Extraction Protocol for NMR and Mass Spectrometry Based Metabolomics, *Anal. Biochem.* **2008**, *372*, 204–212.

[37] X. Liu, L. Zhang, L. You, J. Yu, M. Cong, Q. Wang, F. Li, et al., Assessment of Clam *Ruditapes philippinarum* as Heavy Metal Bioindicators Using NMR-Based Metabolomics, *Clean – Soil Air Water* **2011**, *39*, 759–766.

[38] X. Liu, C. Yang, L. Zhang, L. Li, S. Liu, J. Yu, L. You, et al., Metabolic Profiling of Cadmium-Induced Effects in One Pioneer Intertidal Halophyte *Suaeda salsa* by NMR-Based Metabolomics, *Ecotoxicology* **2011**, *20*, 1422–1431.

[39] X. Liu, L. Zhang, L. You, H. Wu, J. Zhao, M. Cong, F. Li, et al., Metabolomic Study on the Halophyte *Suaeda salsa* in the Yellow River Delta, *Clean – Soil Air Water* **2011**, *39*, 720–727.

[40] F. W. M. T. Fan, Metabolite Profiling by One- and Two-Dimensional NMR Analysis of Complex Mixtures, *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161–219.

[41] M. R. Viant, E. S. Rosenblum, R. S. Tjeerdema, NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health, *Environ. Sci. Technol.* **2003**, *37*, 4982–4989.

[42] P. V. Purohit, D. M. Rocke, M. R. Viant, D. L. Woodruff, Discrimination Models Using Variance-Stabilizing Transformation of Metabolomic NMR Data, *OMICS J. Integr. Biol.* **2004**, *8*, 118–130.

[43] H. M. Parsons, C. Ludwig, U. L. Gunther, M. R. Viant, Improved Classification Accuracy in 1- and 2-Dimensional NMR Metabolomics Data Using the Variance Stabilising Generalised Logarithm Transformation, *BMC Bioinf.* **2007**, *8*, 234.

[44] H. C. Keun, T. M. D. Ebbels, H. Antti, M. E. Bollard, O. Beckonert, E. Holmes, J. C. Lindon, J. K. Nicholson, Improved Analysis of Multivariate Data by Variable Stability Scaling: Application to NMR-Based Metabolic Profiling, *Anal. Chim. Acta* **2003**, *490*, 265–276.

[45] V. G. Tusher, R. Tibshirani, G. Chu, Significance Analysis of Microarrays Applied to the Ionizing Radiation Response, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 5116–5121.

[46] T. D. Williams, H. Wu, E. M. Santos, J. Ball, I. Katsiadaki, M. M. Brown, P. Baker, et al., Hepatic Transcriptomic and Metabolomic

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Responses in the Stickleback (*Gasterosteus aculeatus*) Exposed to Environmentally Relevant Concentrations of Dibenzanthracene, *Environ. Sci. Technol.* **2009**, *43*, 6341–6348.

[47] Y. S. Kim, Malonate Metabolism: Biochemistry, Molecular Biology, Physiology, and Industrial Application, *J. Biochem. Mol. Biol.* **2002**, *35*, 443–451.

[48] D. K. Stumpf, R. H. Burris, Biosynthesis of Malonate in Roots of Soybean Seedlings, *Plant Physiol.* **1981**, *68*, 992–995.

[49] J. G. Bundy, D. Osborn, J. M. Weeks, J. C. Lindon, J. K. Nicholson, An NMR-Based Metabonomic Approach to the Investigation of Coelomic Fluid Biochemistry in Earthworms under Toxic Stress, *FEBS Lett.* **2001**, *500*, 31–35.

[50] R. L. Preston, Transport of Amino Acids by Marine Invertebrates, *Comp. Physiol. Biochem.* **2005**, *265*, 410–421.

[51] K.-H. Carlsson, G. Gade, Metabolic Adaptation of the Horseshoe Crab, *Limulus polyphemus*, during Exercise and Environmental Hypoxia and Subsequent Recovery, *The Biol. Bull.* **1986**, *171*, 217–235.

[52] T. M. Stokes, A. Awapara, Alanine and Succinate as End-Products of Glucose Degradation in the Clam *Rangia cuneata*, *Comp. Biochem. Physiol.* **1968**, *25*, 883–892.

[53] R. A. Graham, W. R. Ellington, Anaerobic Aspartate Metabolism and the Formation of Alanine in Molluscan Cardiac Muscle: A ^{13}C-NMR Study, *J. Exp. Zool.* **1985**, *236*, 365–370.

[54] D. W. Choi, Glutamate Neurotoxicity and Diseases of the Nervous System, *Neuron* **1988**, *1*, 623–634.

[55] B. A. O'Gara, V. K. Bohannon, M. W. Teague, M. B. Smeaton, Copper-Induced Changes in Locomotor Behaviors and Neuronal Physiology of the Freshwater Oligochaete, *Lumbrculus variegates*, *Aquat. Toxicol.* **2004**, *69*, 51–66.

[56] J. H. F. M. Kluytmans, P. R. Veenhof, A. de Zwaan, Anaerobic Production of Volatile Fatty Acids in the Sea Mussel *Mytilus edulis* (L.), *J. Comp. Physiol.* **1975**, *104*, 71–78.

[57] A. de Zwaan, W. J. A. van Marrewijk, Anaerobic Glucose Degradation in the Sea Mussel *Mytilus edulis* L., *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.* **1973**, *44*, 429–439.

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