



Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics

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ABSTRACT

Copper is an important heavy metal contaminant with high ecological risk in the Bohai Sea. In this study, the metabolic responses in the bioindicator, Manila clam (*Ruditapes philippinarum*), to the environmentally relevant copper exposures were characterized using NMR-based metabolomics. The significant metabolic changes corresponding to copper exposures were related to osmolytes, intermediates of the Krebs cycle and amino acids, such as the increase in homarine, branched chain amino acids and decrease in succinate, alanine and dimethylamine in the copper-exposed clam gills during 96 h exposure period. Overall, Cu may lead to the disturbances in osmotic regulation and energy metabolism in clams during 96 h experimental period. These results demonstrate that NMR-based metabolomics is applicable for the discovery of metabolic biomarkers which could be used to elucidate the toxicological mechanisms of marine heavy metal contaminants.

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1. Introduction

Copper (Cu) is an essential element for organisms, since it is a cofactor of the prion protein and many enzymes. It acts as a catalyst for many enzyme systems, and is important as an electron carrier in intracellular structures (Hay, 1984). However, excessive copper is highly toxic due to its high affinity for thiol groups. Cu can participate in redox reactions that generate highly reactive hydroxyl radical enhancing the production of reactive oxygen species (ROS) through Fenton reactions associated with free Cu to catalyze the reaction between superoxide anion and H₂O₂ and direct binding to free thiols of cysteines, which can cause catastrophic damage to lipids, proteins and DNA (Halliwell, 1999; Ceconi et al., 2002). Copper pollution along the Bohai Sea mainly arises from mining and electroplating, which has posed severe ecological risk on the marine ecosystem of the Bohai Sea (Ma et al.,

1995). Ma et al. reported that excessive copper had become one of most severe pollutants in the Jinzhou Bay of Bohai Sea due to the high levels of copper accumulated in the invertebrates listed in the “Mussel Watch Program” (Ma et al., 1995). It is therefore necessary to assess the toxicological effects and subsequent ecological risk of copper in the marine and coastal environments.

Marine bivalves can accumulate heavy metals in direct proportion to their environmental levels (Roesijadi, 1980). Therefore, marine mussels and oysters have often been used as sentinel organisms in many countries for heavy metal pollution monitoring since “Mussel Watch Program” was proposed in the late 1970s (Goldberg, 1975). Manila clam, *Ruditapes philippinarum*, is consumed as economic seafood and distributed in the natural environment along the coasts of the Bohai Sea. It has been considered a good sentinel organism for the heavy metal pollution monitoring of marine and coast ecosystems and assigned as a bioindicator in the marine ecotoxicology due to its wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of heavy metals (Park et al., 2006; Park and Tsutsumi, 2008; Ji et al., 2006).

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Traditional toxicological studies on heavy metals have usually focused on the measurement of specific responses such as the activity of acetylcholinesterase to test for neuro-toxicity or antioxidant enzyme levels to test for oxidative stress induced by heavy metals (Matozzo et al., 2005; Elbaz et al., 2010; Geret et al., 2002). Therefore, some of these enzymatic parameters such as activity of acetylcholinesterase have been used as biomarkers for the heavy metal monitoring (Matozzo et al., 2005). With the development of system biology, metabolomics, one of the techniques of system biology, has been widely applied in drug toxicity studies, disease diagnosis, functional genomics and environmental sciences (Wu et al., 2005; Waters et al., 2001; Marchesi et al., 2007; Gavaghan et al., 2002; Griffin et al., 2004; Brindle et al., 2002; Bundy et al., 2004; Viant et al., 2006a,b; Katsiadaki et al., 2009). The application of proton nuclear magnetic resonance (NMR) spectroscopy combined with pattern recognition methods to detect the responses of low molecular weight metabolites (<1000 Da) to toxicants or environmental contaminants has been demonstrated in both terrestrial vertebrate and invertebrate systems relevant to environmental toxicology (Bundy et al., 2002; Griffin et al., 2000; Fiehn, 2002). ^1H NMR spectroscopy is suitable for the detection of a large range of endogenous low molecular weight metabolites in an organ or cells, since practically all metabolite molecules contain protons. Additionally, NMR is a rapid, non-destructive analytical approach that delivers rich structural and quantitative information and can allow the metabolites to be analyzed simultaneously (Jones et al., 2008; Tuffnail et al., 2009; Viant et al., 2003).

Gill tissue is a main target tissue for heavy metal accumulation in marine bivalve invertebrates (Panfoli et al., 2000; Viarengo et al., 1994). Hence, it is potentially sensitive and suitable for the detection of metabolic biomarkers induced by accumulated heavy metal. In this study, therefore, ^1H NMR-based metabolomics was applied to the gill tissue extracts from Manila clam *R. philippinarum*, one of the bioindicators for marine heavy metal monitoring, to detect the metabolic responses in gill tissues as to copper exposure. Previously, Jones et al., (2008) reported the toxicological effects induced by nickel at the concentration of EC_{50} in blue mussel (*Mytilus galloprovincialis*). However, the concentration ($770 \mu\text{g L}^{-1}$) of heavy metal was not environmentally relevant. In this work, the aim was to detect metabolic biomarkers characterizing the toxicological effects of copper with environmentally relevant concentrations in adult Manila clams with various exposure times.

2. Materials and methods

2.1. Sample collection

All the adult Manila clams *R. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local culturing farm. Animals were allowed to acclimate in aerated seawater (25°C , 32 psu, collected from clean environment) in the laboratory for 10 days and fed with the *Chlorella vulgaris* Beij daily. After acclimatization, a total 60 clams were randomly divided into 3 flat-bottomed rectangular tanks with 20 individual animals respectively.

For the challenge experiment, clams were exposed with copper at following concentrations, 10 and $40 \mu\text{g L}^{-1}$ (as CuCl_2 , 10 and $40 \mu\text{g L}^{-1}$). The concentrations of Cu can be found in some heavily polluted sites of Bohai Sea (Ma et al., 1995). Clams cultured in the normal fresh seawater (FSW) were used as control samples. The gill tissues of five clams from each tank were randomly sampled for metabolomics analysis after exposure for 24, 48 and 96 h respectively. After collection, the 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 methanol/chloroform solvent system (Bligh and Dyer, 1959; Lin et al., 2007; Wu et al., 2008). Briefly, the gill tissue ($\sim 100 \text{ mg}$) was homogenized in 4 mL g^{-1} (solvent volume/tissue mass) of methanol and 0.85 mL g^{-1} of water using a high throughput homogenizer, Precellys 24 (Bertin, France). The homogenate was then 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 homogenate, and the mixture was vortexed and centrifuged (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 were subsequently resoluted in $600 \mu\text{L}$ 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-d4-propionate (TSP) as chemical shift standard in D_2O . The mixture was vortexed and then centrifuged at 2500 g for 5 min at 4°C . The supernatant ($550 \mu\text{L}$) was pipetted into a 5 mm NMR tube prior to NMR measurement.

2.3. NMR spectroscopy

The gill tissue extracts were analyzed using a Bruker AV 500 NMR spectrometer operated at 500.18 MHz at 298 K . Basic one-dimensional (1-D) ^1H NMR spectra were obtained using a $11.9 \mu\text{s}$ 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. Data sets 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 (Fan, 1996; Viant et al., 2003) and using the software, Chenomx (Evaluation Version, Chenomx Inc., Canada). Some of the metabolites were confirmed by the 2D NMR method, ^1H – ^1H homonuclear correlation spectroscopy (COSY).

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 (version 7.0; The MathWorks, Natick, MA) (Purohit et al., 2004). 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, $\lambda = 1 \times 10^{-8}$) to stabilize the variance across the spectral bins and to enhance the weightings of the less intense peaks (Purohit et al., 2004; Parsons et al., 2007). The data sets were preprocessed using mean-centering before either principal components analysis (PCA) or partial least-squares discriminant analysis (PLS-DA) was applied using PLS Toolbox software (version 4.0, Eigenvector Research, Manson, WA).

Two well-developed pattern recognition methods, PCA and PLS-DA, were used in this work to separate the sample groups. PCA is an exploratory unsupervised pattern recognition 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. The algorithm of this pattern recognition method calculates the highest amount of correlated variation along PC1, with subsequent PCs containing

correspondingly smaller amounts of variance. For each model built, the loading vector for the PC could be examined to identify the metabolites which contributed to the clusters. PLS-DA is a supervised pattern recognition technique and is frequently used to classify multiple classes by searching for variables (the *X* matrix) which are correlated with class membership (the *Y* matrix). In PLS-DA, the *X* matrix is the measured matrix, i.e., the NMR data, and the *Y* matrix is composed of dummy variables (represented by ones and zeros) that indicate the class for each treatment (Keun et al., 2003). The prediction accuracy of the PLS-DA model was assessed using cross-validation with leave-one-out (Rubingh et al., 2006; Westerhuis et al., 2008). A Q^2 score > 0.08 indicates that the model classification is significantly better than chance, while a score greater than 0.4 indicates that the model is practically robust (Lindon et al., 1999). Similar to PCA, the weights vector for the Latent Variables (LV) in PLS-DA can be applied for the identification of these potentially significant metabolites which contribute to the clusters due to the biological variations between control and exposed groups. One way ANOVA (analysis of variance) with Tukey's test was performed on the ratio of representative bin area (peak intensity) to the total spectral area which was contributive for the separation between control and copper-treated samples and was used as the "concentration" of corresponding metabolite. A *P* value of 0.05 was considered significant for ANOVA on the metabolites between control and exposed samples.

3. Results

3.1. ^1H NMR spectroscopy of gill tissue extracts

A representative ^1H NMR spectrum of gill tissue extracts from a control clam is shown in Fig. 1. Several metabolite classes were identified, including amino acids (branched chain amino acids: valine, leucine and isoleucine, arginine, glutamate, glycine, etc.), energy storage compounds (α , β -glucose, ATP/ADP and glycogen), organic osmolytes (betaine, homarine, etc.), and Krebs cycle intermediates (succinate, citrate and α -ketoglutarate). However, the original NMR spectrum (Fig. 1A) is dominated by the key organic osmolyte, betaine (3.25 and 3.91 ppm), which is 10–100 times higher in the intensity than other metabolites.

3.2. Pattern recognitions on the ^1H NMR spectra of gill tissue extracts

Principal components analysis was initially applied to the NMR spectral data sets of gill tissue extracts after 24, 48 and 96 h exposures. However, no significant difference ($P > 0.05$) along various principal components between the control and exposed groups was detected using one way analysis of variance (ANOVA) on the PC scores (data not shown). Hence the supervised technique PLS-DA was employed to classify the control and exposure groups (Figs. 2–4). PLS-DA resulted in clear separation between the groups (Figs. 2–4A) with Q^2 values more than 0.4 showing the high robustness of PLS models and was therefore used throughout the rest of this study. After 24 h of exposure, the gill samples from control and low ($10 \mu\text{g L}^{-1}$) Cu-exposed groups were separated mainly along LV1, while the control and high ($40 \mu\text{g L}^{-1}$) Cu-exposed groups were tightly clustered along LV2 (Fig. 2A). The scores plot (LV1 vs. LV2) showed clear separation between control and Cu-treated groups after exposure for 48 h. However, there was no separation between low and high Cu-treated samples (Fig. 3A). For these clam gill samples after 96 h of exposure, the control and low ($10 \mu\text{g L}^{-1}$) Cu-exposed groups were clearly separated along LV2, and the separation between control and high ($40 \mu\text{g L}^{-1}$) Cu-exposed groups was found along LV1 (Fig. 4A).

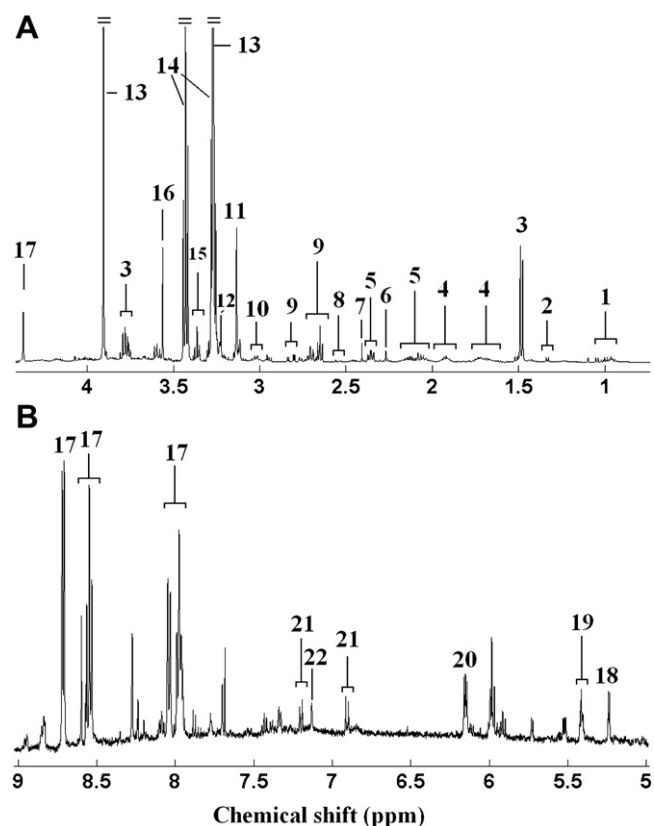


Fig. 1. A representative one-dimensional 500 MHz ^1H NMR spectrum of gill tissue extracts from a control clam (A) vertical expansion of the aromatic region (B). Keys: (1) Branched chain amino acids: isoleucine, leucine and valine, (2) lactate, (3) alanine, (4) arginine, (5) glutamate, (6) acetoacetate, (7) succinate, (8) citrate, (9) aspartate, (10) α -ketoglutarate, (11) malonate, (12) acetylcholine, (13) betaine, (14) taurine, (15) hypotaurine (16) glycine, (17) homarine, (18) glucose, (19) glycogen, (20) ATP/ADP, (21) tyrosine and (22) histidine.

The plots (Fig. 2B and C, Fig. 3B, Fig. 4B and C) of either LV1 or LV2 weights were originally used to identify the NMR spectral bins of metabolites which might be significant for the separation between the control and exposed groups. Then, one way analysis of variance with a 5% significance level was applied to the NMR spectral bin areas that presented a possible contribution to the separation, facilitating the identification of metabolic changes.

Basically, the levels of aspartate, dimethylamine and hypotaurine were significantly elevated in the gill tissues from the low ($10 \mu\text{g L}^{-1}$) dose group after 24 h of exposure, while alanine, acetate, succinate, citrate, acetylcholine were decreased significantly (Table 1, Fig. 2B). For high dose ($40 \mu\text{g L}^{-1}$) of exposure, the metabolic responses in clam gill tissues included significantly increased aspartate, betaine, homarine and glycogen, and significantly decreased alanine, acetate, succinate, citrate, dimethylamine and acetylcholine (all significant at the 5% level) (Table 1, Fig. 2C). After 48 h of Cu exposures, the most significant metabolic changes in gill tissues were the elevation of branched chain amino acids, citrate, betaine, glycine and homarine, and decreased levels of alanine, acetate, dimethylamine, aspartate, succinate, acetylcholine and ATP/ADP from both low and high Cu-dosed samples (Table 1, Fig. 3B). The metabolic profiles resulted in significant increase in alanine and hypotaurine, and decrease in betaine, taurine and acetate in the low ($10 \mu\text{g L}^{-1}$) Cu-dosed samples after exposure for 96 h, together with the elevated branched chain amino acids, hypotaurine and homarine and depleted acetate, dimethylamine, betaine and taurine in the high ($40 \mu\text{g L}^{-1}$) Cu-dosed samples (Table 1, Fig. 4B and C).

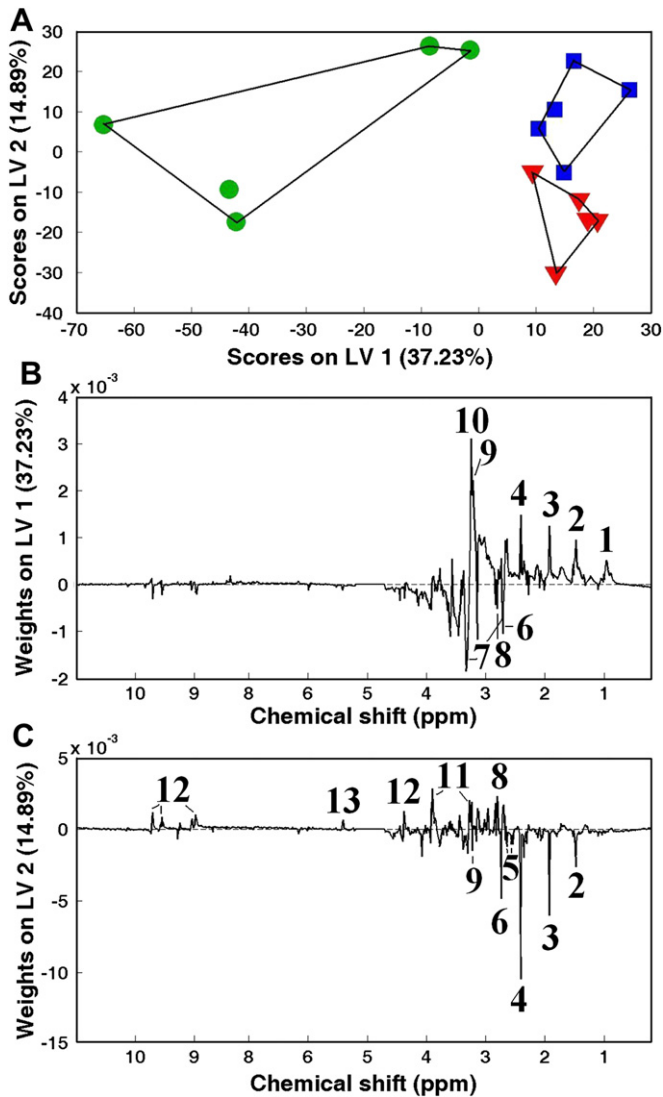


Fig. 2. Partial Least-Squares Discriminant Analysis (PLS-DA) model showing (A) separations between control (▼), 10 (●) and 40 $\mu\text{g L}^{-1}$ Cu-exposed (■) clam samples after exposure for 24 h ($Q^2 = 0.57$); and corresponding LV1 (B) and LV2 (C) weights plots showing the metabolic differences between the groups. Keys: (1) Branched chain amino acids: isoleucine, leucine and valine, (2) alanine, (3) acetate, (4) succinate, (5) citrate, (6) dimethylamine, (7) hypotaurine, (8) aspartate, (9) acetylcholine, (10) unknown (3.24 ppm), (11) betaine, (12) homarine and (13) glycogen.

4. Discussion

Metabolomics is a well-established technique of system biology based on the measurement and analysis of low molecular weight endogenous metabolites using modern analytical techniques including NMR spectroscopy and Mass spectrometry (Wang et al., 2003; Plumb et al., 2003). The metabolic profiling can provide an overview of the metabolic status of a biological system including cells, tissues, organs or even whole organisms (Lin et al., 2006; Stentiford et al., 2005).

After PLS-DA analysis, the LV score plot showed clear separations between the control and Cu-exposed groups (10 and 40 $\mu\text{g L}^{-1}$) after 24 h of exposure (Fig. 2A), which meant the metabolic differences between various Cu-dosed clam gill samples. Although common metabolic changes in both low and high Cu-exposed groups were detected including the elevated aspartate and decreased alanine, acetate, succinate, citrate and acetylcholine,

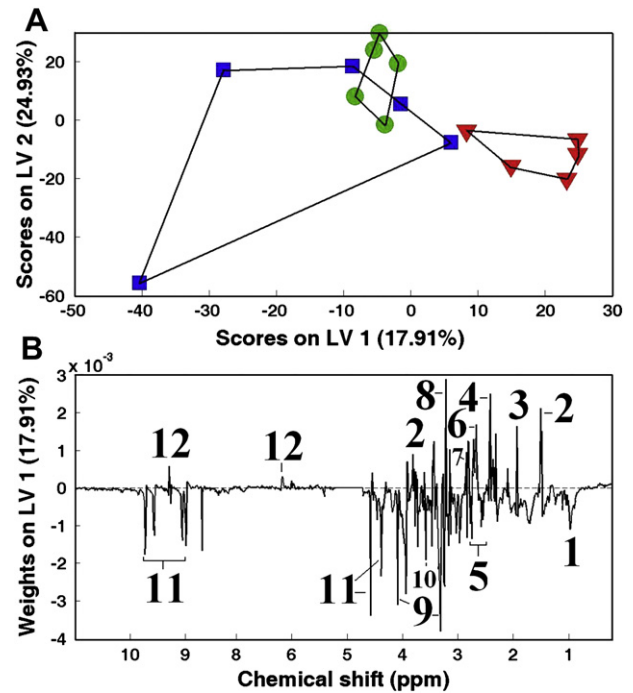


Fig. 3. Partial Least-Squares Discriminant Analysis (PLS-DA) model showing (A) separations between control (▼), 10 (●) and 40 $\mu\text{g L}^{-1}$ Cu-exposed (■) clam samples after exposure for 48 h ($Q^2 = 0.49$); and corresponding LV1 (B) weights plots showing the metabolic differences between the groups. Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) alanine, (3) acetate, (4) succinate, (5) citrate, (6) dimethylamine, (7) aspartate, (8) acetylcholine, (9) betaine, (10) glycine, (11) homarine and (12) ATP/ADP.

there were a few specific metabolic differences between these 2 dosed groups such as the elevated hypotaurine in low Cu-dosed samples and elevated betaine, homarine and glycogen and decreased dimethylamine in high Cu-dosed samples. All these findings implied the differential metabolic responses induced by various doses of Cu in the clam gill samples after exposure for 24 h. After 48 h of Cu exposures, both low and high doses of Cu-exposed samples were classified from controls along negative LV1. However, there was no separation between low and high Cu-treated samples, which indicated the similar metabolic responses induced by either low or high dose of Cu after 48 h of exposure (Fig. 3A). The separation between control and low (10 $\mu\text{g L}^{-1}$) Cu-exposed groups after exposure for 96 h were found along LV2, while the high (40 $\mu\text{g L}^{-1}$) Cu-exposed groups were classified from the controls along LV1 (Fig. 4A). It indicated the distinguishable metabolic responses in the gill tissues from either low or high dose of Cu-exposed groups from the controls due to the differential metabolic biomarkers (Fig. 4B and C).

The original NMR spectrum (Fig. 1A) was dominated by a couple of organic osmolytes, betaine (3.27 and 3.91 ppm) and taurine (3.27 and 3.45 ppm) (Fig. 1A), which were 10–100 times higher in NMR peak intensities than other metabolites. Organic osmolytes such as betaine, homarine, hypotaurine, dimethylamine, alanine and taurine are small organic molecules functioning in the osmotic regulation in marine organisms via various metabolic pathways (Preston, 2005). The osmolytes can be actively accumulated or released when the salinity increase or decreases. Therefore, organic osmolytes play key physiological roles in osmotic regulation of invertebrates and were therefore detected at higher levels than other metabolites in clams (Preston, 2005). In the clam gills after 24 h of exposure of Cu, the elevation of hypotaurine and reduction of alanine in low (10 $\mu\text{g L}^{-1}$) dose of Cu-treated samples exhibited

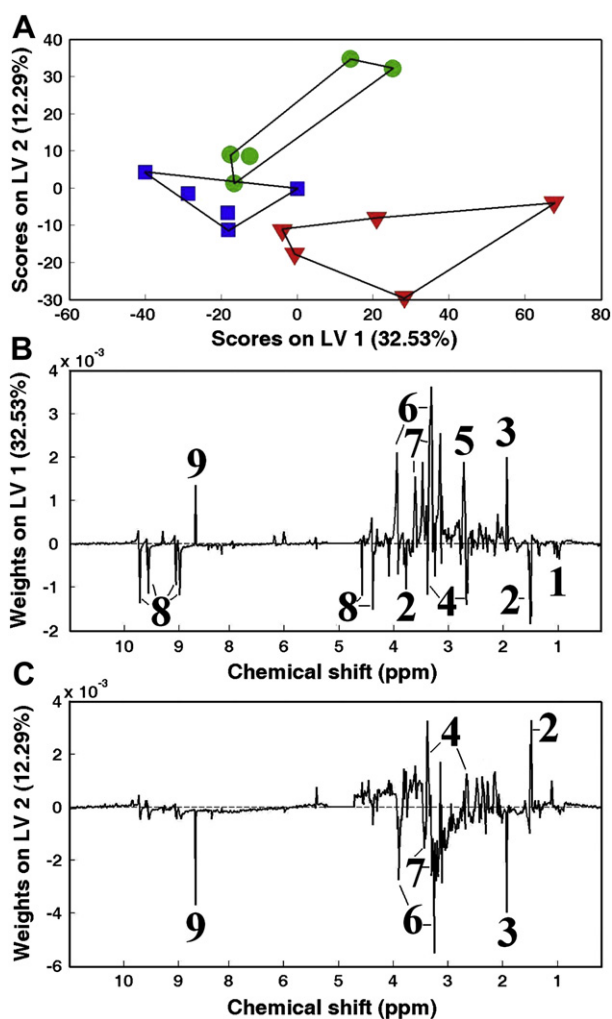


Fig. 4. Partial Least-Squares Discriminant Analysis (PLS-DA) model showing (A) separations between control (\blacktriangledown), $10 \mu\text{g L}^{-1}$ (\bullet) and $40 \mu\text{g L}^{-1}$ Cu-exposed (\blacksquare) clam samples after exposure for 96 h ($Q^2 = 0.50$); corresponding LV1 (B) and LV2 (C) weights plots showing the metabolic differences between the groups. Keys: (1) Branched chain amino acids: isoleucine, leucine and valine, (2) lactate, (3) alanine, (4) acetate, (5) dimethylamine, (6) betaine, (7) taurine, (8) homarine and (9) unknown (7.68 ppm).

the disturbance in osmotic regulation in clam gills. However, the differential metabolic changes including the increased levels of betaine and homarine and the decreased levels of alanine in high ($40 \mu\text{g L}^{-1}$) dose of Cu-treated samples might indicate the disturbance in osmotic regulation via various metabolic pathways compared with that in low ($10 \mu\text{g L}^{-1}$) dose of Cu-treated samples as mentioned above. It demonstrated the dose-responsive toxicological effects induced by various doses of Cu.

It has been reported that alanine also constitutes the major portion of end-product of glucose breakdown anaerobically, together with the metabolite of succinate in invertebrate (Carlsson and Gade, 1986; Stokes and Awapara, 1968). However, both succinate and alanine were down-regulated in this work after exposure of Cu for 24 h. In another possible metabolic pathway, there is substantial conversion of aspartate to succinate with no detectable enrichment of other compounds under anoxic conditions in mollusk hence with the decreased aspartate and increased succinate (Graham and Ellington, 1985). Interestingly, the contrary result of increased aspartate as well as decreased succinate was found in the copper-exposed clams in our case, which meant no anaerobiosis metabolism induced by Cu in clam gills. Therefore the decrease

Table 1

Significantly up- or down-regulated metabolites involved in various physiological functions ($P < 0.05^a$) in gill tissues of Cu-exposed clams after exposures for 24, 48 and 96 h.

Dose	$10 \mu\text{g Cu}^{2+} \text{ L}^{-1}$ seawater	$40 \mu\text{g Cu}^{2+} \text{ L}^{-1}$ seawater
24 h	<i>Osmotic regulation:</i>	<i>Osmotic regulation:</i>
	Aspartate \uparrow	Aspartate \uparrow
	Dimethylamine \uparrow	Betaine \uparrow
	Hypotaurin \uparrow	Homarine \uparrow
	Alanine \downarrow	Alanine \downarrow
	<i>Energy metabolisms:</i>	Acetate \downarrow
	Acetate \downarrow	Glycogen \uparrow
	Succinate \downarrow	Dimethylamine \downarrow
	Citrate \downarrow	<i>Energy metabolisms:</i>
	<i>Miscellaneous:</i>	Succinate \downarrow
	Acetylcholine \downarrow	Citrate \downarrow
		<i>Miscellaneous:</i>
48 h	<i>Osmotic regulation:</i>	<i>Osmotic regulation:</i>
	Branched chain amino acids \uparrow	Branched chain amino acids \uparrow
	Citrate \uparrow	Betaine \uparrow
	Betaine \uparrow	Glycine \uparrow
	Glycine \uparrow	Homarine \uparrow
	Homarine \uparrow	Alanine \downarrow
	Alanine \downarrow	Dimethylamine \downarrow
	Dimethylamine \downarrow	Aspartate \downarrow
	<i>Energy metabolisms:</i>	<i>Energy metabolisms:</i>
	Acetate \downarrow	Acetate \downarrow
	Aspartate \downarrow	Succinate \downarrow
	Succinate \downarrow	Citrate \uparrow
ATP/ADP \downarrow	ATP/ADP \downarrow	
<i>Miscellaneous:</i>	<i>Miscellaneous:</i>	
Acetylcholine \downarrow	Acetylcholine \downarrow	
96 h	<i>Osmotic regulation:</i>	<i>Osmotic regulation:</i>
	Alanine \uparrow	Branched chain amino acids \uparrow
	Hypotaurine \uparrow	Hypotaurine \uparrow
	Betaine \downarrow	Homarine \uparrow
	Taurine \downarrow	Betaine \downarrow
	<i>Energy metabolisms:</i>	Taurine \downarrow
	Acetate \downarrow	Dimethylamine \downarrow
		<i>Energy metabolisms:</i>
		Acetate \downarrow

^a P values determined using One way ANOVA on the bin areas from the representative peak of corresponding metabolite.

of succinate and citrate in both low and high doses-exposed groups meant the possible perturbations in energy metabolism caused by both low and high doses of Cu after 24 h of exposure.

Acetylcholine is a neural transmitter that can be degraded to choline in cholinergic synapses and neuromuscular junctions by acetylcholinesterase (AChE) (Matozzo et al., 2005) 8568; et al., on. In some studies, the measurement of AChE activity was demonstrated useful as a biomarker of neurotoxic compounds in aquatic organisms and has been successfully applied for the monitoring of neurotoxic contaminants (Matozzo et al., 2005; Cajaraville et al., 2000). As a matter of fact, the inhibition of AChE is followed by accumulation of acetylcholine, however, the significant decrease of acetylcholine was detected in clam gills caused by both low and high doses of Cu after 24 h of exposure. In our previous work, the accumulation of acetylcholine was detected in gill tissues of clam after exposure with mercury that is known as a typical neurotoxic heavy metal (Liu et al., 2011). Elevated acetylcholine was then recognized as the biomarker of neuro-toxicity induced by mercury (Liu et al., 2011). In this work, it seemed copper exposures could induce contrary alteration in acetylcholine to that caused by mercury exposure. The physiological mechanism was unclear and needed further studies.

Acetate is an end-product of anaerobic metabolism that can be used as a biomarker of exposure to anoxic conditions with the elevated levels in the biological tissues (Kluytmans et al., 1975; Zwaan and Marrewijk et al., 1973). In this work, the level of

acetate was significantly reduced in clam gill tissues after exposed to Cu for 24 h, which might imply the reduced anaerobic metabolism in gills.

Compared to the metabolic changes induced by Cu exposures after 24 h, the specific metabolic responses including increased branched chain amino acids and glycine and decreased ATP/ADP were observed in both low and high doses of Cu-exposed samples after 48 h of exposure. Recent studies have reported that some marine mollusks use high intracellular concentrations of free amino acids to balance their intracellular osmolarity with the environment, and these pools of oxidizable amino acids were also used extensively in cellular energy metabolism (Viant et al., 2003). The increase of amino acids in clam gill tissues indicated the disturbances in osmolarity and energy metabolism caused by Cu exposures.

After exposed to Cu for 96 h, the levels of branched chain amino acids, succinate, citrate, acetylcholine, aspartate in the low ($10 \mu\text{g L}^{-1}$) dose of Cu-exposed samples were recovered to the control levels, which meant the recovery of disturbances in energy metabolism and osmotic regulations due to the biological adaptation to the low dose of Cu exposure. On the other hand, the unique metabolic responses including the increased betaine and taurine (2 organic osmolytes) were detected after exposure to low dose of Cu for 96 h. For the high dose ($40 \mu\text{g L}^{-1}$) of Cu-treated samples, the levels of citrate, succinate, acetylcholine, glycine and ATP returned to the control levels as well. However, the osmolyte, taurine was decreased like that in the low dose of Cu-treated samples. It indicated the time-dependent responses of new disturbances in the osmotic regulation based on the alterations in the levels of betaine and/or taurine induced by either low ($10 \mu\text{g L}^{-1}$) or high ($40 \mu\text{g L}^{-1}$) dose of Cu exposure for 96 h.

In summary, this work examined the metabolic responses and toxicological effects in the gill tissues of Manila clam *R. philippinarum* induced by two doses (10 and $40 \mu\text{g L}^{-1}$) of Cu during 96 h of exposure. The dose- and time-dependent effects of Cu mainly included the disturbances in energy metabolisms and osmotic regulations based on the corresponding metabolic biomarkers such as succinate, citrate, ATP, branched chain amino acids, betaine, taurine, hypotaurine, glycine, aspartate, acetylcholine, homarine and dimethylamine. Our findings demonstrated the applicability of metabolomics for the elucidation of toxicological effects of heavy metals in the bioindicator and the potential of metabolic biomarkers for the monitoring of heavy metal contaminants in the marine environment.

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References

- Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37, 911–917.
- Brindle, J.T., Antti, H., Holmes, E., Tranter, G., Nicholson, J.K., Bethell, H.W.L., Clarke, S., Schofield, P.M., McKilligan, E., Mosedale, D.E., Grainger, D.J., 2002. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using $^1\text{H-NMR}$ -based metabolomics. *Nature Medicine* 8, 1439–1444.
- Bundy, J.G., Lenz, E.M., Bailey, N.J., Gavaghan, C.L., Svendsen, C., Spurgeon, D., Hankard, P.K., Osborn, D., Weeks, J.M., Trauger, S.A., Apeir, P., Sanders, I., Lindon, J.C., Nicholson, J.K., Tang, H., 2002. 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. *Environmental Toxicology and Chemistry* 21, 1966–1972.
- Bundy, J.G., Spurgeon, D.J., Svendsen, C., Hankard, P.K., Weeks, J.M., Osborn, D., Lindon, J.C., Nicholson, J.K., 2004. Environmental metabolomics: applying combination biomarker analysis in earthworms at a metal contaminated site. *Ecotoxicology* 2004 (13), 797–806.
- Cajarville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *The Science of the Total Environment* 247, 295–311.
- Carlsson, K.-H., Gade, G., 1986. Metabolic adaptation of the horseshoe crab, *Limulus polyphemus*, during exercise and environmental hypoxia and subsequent recovery. *The Biological Bulletin* 171, 217–235.
- Cecconi, I., Scaloni, A., Rastelli, G., Moroni, M., Vilaro, P.G., Costantino, L., Cappiello, M., Garland, D., Carper, D., Petrash, J.M., Del Corso, A., Mura, U., 2002. Oxidative modification of aldose reductase induced by copper ion: definition of the metal-protein interaction mechanism. *Journal of Biological Chemistry* 277, 42017–42027.
- De Zwaan, A., Van Marrewijk, W.J.A., 1977. Anaerobic glucose degradation in the sea mussel *Mytilus edulis* L. *Comparative Biochemistry and Physiology B*, 429–439.
- Elbaz, A., Wei, Y.Y., Meng, Q., Zheng, Q., Yang, Z.M., 2010. Mercury-induced oxidative stress and impact on antioxidant enzymes in *Chlamydomonas reinhardtii*. *Ecotoxicology* 19, 1285–1293.
- Fan, W.M.T., 1996. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Progress in Nuclear Magnetic Resonance Spectroscopy* 28, 161–219.
- Fiehn, O., 2002. Metabolomics: the link between genotypes and phenotypes. *Plant Molecular Biology* 48, 155–171.
- Gavaghan, C.L., Wilson, I.D., Nicholson, J.K., 2002. Physiological variation in metabolic phenotyping and functional genomic studies: use of orthogonal signal correction and PLS-DA. *FEBS Letters* 530, 191–196.
- Geret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gills of the clam *Ruditapes decussates*. *Marine Environmental Research* 54, 413–417.
- Goldberg, E.D., 1975. The mussel watch: a first step in global marine pollution monitoring. *Marine Pollution Bulletin* 6, 111.
- Graham, R.A., Ellington, W.R., 1985. Anaerobic aspartate metabolism and the formation of alanine in molluscan cardiac muscle: a ^{13}C NMR study. *Journal of Experimental Zoology* 236, 365–370.
- Griffin, J.L., Cemal, C.K., Pook, M.A., 2004. Defining a metabolic phenotype in the brain of a transgenic mouse model of spinocerebellar ataxia 3. *Physiological Genomics* 16, 334–340.
- Griffin, J.L., Walker, L.A., Garrod, S., Holmes, E., Shore, R.F., Nicholson, J.K., 2000. NMR spectroscopy based metabolomics 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. *Comparative Biochemistry and Physiology B* 127, 357–367.
- Halliwell, B., 1999. *Free Radicals in Biology and Medicine*. Oxford University Press, New York.
- Hay, R.W., 1984. *Bio-Inorganic Chemistry*. Ellis Horwood, Camelot Press, Great Britain.
- Ji, J., Choi, H.J., Ahn, I.-Y., 2006. 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. *Marine Pollution Bulletin* 52, 447–468.
- Jones, O.A.H., Dondero, F., Viarengo, A., Griffin, J.L., 2008. Metabolic profiling of *Mytilus galloprovincialis* and its potential applications for pollution assessment. *Marine Ecology Progress Series* 369, 169–179.
- Katsiadaki, I., Williams, T.D., Ball, J.S., Bean, T.P., Sanders, M.B., Wu, H., Santos, E.M., Brown, M.M., Baker, P., Ortega, F., Falciani, F., Craft, J.A., Tyler, C.R., Viant, M.R., Chipman, J.K., 2009. Hepatic transcriptomic and metabolomic responses in the Stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. *Aquatic Toxicology* 43, 6341–6348.
- Keun, H.C., Ebbels, T.M.D., Antti, H., Bollard, M.E., Beckonert, O., Holmes, E., Lindon, J.C., Nicholson, J.K., 2003. Improved analysis of multivariate data by variable stability scaling: application to NMR-based metabolic profiling. *Analytica Chimica Acta* 490, 265–276.
- Kluytmans, J.H.F.M., Veenhof, P.R., De Zwaan, A., 1975. Anaerobic production of volatile fatty acids in the sea mussel *Mytilus edulis* (L.). *Journal of Comparative Physiology* 104, 71–78.
- Lin, C.Y., Viant, M.R., Tjeerdema, R.S., 2006. Metabolomics: methodologies and applications in the environmental sciences. *Journal of Pesticide Science* 31, 245–251.
- Lin, C.Y., Wu, H., Tjeerdema, R.S., Viant, M.R., 2007. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics* 3, 55–67.
- Lindon, J.C., Nicholson, J.K., Everett, J.R., 1999. NMR spectroscopy of biofluid. *Annual Reports on NMR Spectroscopy* 38, 1–88.
- Liu, X., Zhang, L., You, L., Yu, J., Zhao, J., Li, L., Wang, Q., Li, F., Li, C., Liu, D., Wu, H., 2011. Differential toxicological effects induced by mercury in gills from three

- pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. *Ecotoxicology* 20, 177–186.
- Ma, J., Shao, M., Bao, Y., Yin, J., 1995. Current status and assessment of Bays of Bohai Sea and Huanghai Sea in Liaoning Province. *Research of Environmental Sciences* 8, 27–34.
- Marchesi, J.R., Holmes, E., Khan, F., Kochhar, S., Scanlan, P., Shanahan, F., Wilson, I.D., Wang, Y.L., 2007. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. *Journal of Proteome Research* 6, 546–551.
- Matozzo, V., Tomei, A., Marin, M., 2005. Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. *Marine Pollution Bulletin* 50, 1686–1693.
- Panfoli, I., Burlando, B., Viarengo, A., 2000. Effects of heavy metals on phospholipase C in gill and digestive gland of the marine mussel *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology B* 127, 391–397.
- Park, K.-I., Paillard, C., Chevalier, P.L., Choi, K.-S., 2006. Report on the occurrence of brown ring disease (BRD) in Manila clam, *Ruditapes philippinarum*, on the west coast of Korea. *Aquaculture* 255, 610–613.
- Park, K.-I., Tsutsumi, H., Hong, J.-S., Choi, K.-S., 2008. Pathology survey of the short-neck clam *Ruditapes philippinarum* occurring on sandy tidal flats along the coast of Ariake Bay, Kyushu, Japan. *Journal of Invertebrate Pathology* 99, 212–219.
- Parsons, H.M., Ludwig, C., Gunther, U.L., Viant, M.R., 2007. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. *BMC Bioinformatics* 8, 234–249.
- Plumb, R.S., Stumpf, C.L., Granger, J.H., Castro-Perez, J., Haselden, J.N., Dear, G.J., 2003. Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Communications in Mass Spectrometry* 2003 (17), 2632–2638.
- Preston, R.L., 2005. Transport of amino acids by marine invertebrates. *Comparative Physiology and Biochemistry* 265, 410–421.
- Purohit, P.V., Rocke, D.M., Viant, M.R., Woodruff, D.L., 2004. Discrimination models using variance stabilizing transformation of metabolomic NMR data. *OMICS: A Journal of Integrative Biology* 8, 118–130.
- Roesijadi, G., 1980. Influence of copper on the clam *Protothaca staminea*: effects on the gills and occurrence of copper-binding proteins. *Biological Bulletin* 158, 233–247.
- Rubingh, C.M., Bijlsma, S., Derks, E.P.A., Bobeldijk, I., Verheij, E.R., Kochhar, S., Smilde, A.K., 2006. Assessing the performance of statistical validation tools for megavariate metabolomics data. *Metabolomics* 2, 53–61.
- Stentiford, G.D., Viant, M.R., Ward, D.G., Johnson, P.J., Martin, A., Wenbin, W., Cooper, H.J., Lyons, B.P., Feist, S.W., 2005. Liver tumors in wild flatfish: a histopathological, proteomic, and metabolomic study. *OMICS: A Journal of Integrative Biology* 9, 281–299.
- Stokes, T.M., Awapara, A., 1968. Alanine and succinate as end-products of glucose degradation in the clam *Rangia cuneata*. *Comparative Biochemistry and Physiology* 25, 883–892.
- Tuffnail, W., Mills, G.A., Cary, P., Greenwood, R., 2009. An environmental ^1H NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation. *Metabolomics* 5, 33–43.
- Viant, M.R., Pincetich, C.A., Hinton, D.E., Tjeerdema, R.S., 2006. Toxic actions of dinoseb in medaka (*Oryzias latipes*) embryos as determined by in vivo ^{31}P NMR, HPLC–UV and ^1H NMR metabolomics. *Aquatic Toxicology* 76, 329–342.
- Viant, M.R., Pincetich, C.A., Hinton, D.E., Tjeerdema, R.S., 2006. Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) determined by ^1H NMR metabolomics. *Aquatic Toxicology* 77, 359–371.
- Viant, M.R., Rosenblum, E.S., Tjeerdema, R.S., 2003. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science and Technology* 37, 4982–4989.
- Viarengo, A., Arena, N., Canesi, L., Alia, F.A., Orunesu, M., 1994. Structural and biochemical alterations in the gills of copper-exposed mussels. In: Renzoni, A., Mattei, N., Lari, L., Fossi, M.-C. (Eds.), *Contaminants in the Environment*. Lewis Publishers, Boca Raton, pp. 135–144.
- Wang, Y., Bollard, M.E., Keun, H., Antti, H., Beckonert, O., Ebbels, T.M., Lindon, J.C., Holmes, E., Tang, H., Nicholson, J.K., 2003. Spectral editing and pattern recognition methods applied to high-resolution magic-angle spinning ^1H nuclear magnetic resonance spectroscopy of liver tissues. *Analytical Biochemistry* 323, 26–32.
- Waters, N.J., Holmes, E., Williams, A., Waterfield, C.J., Farrant, R.D., Nicholson, J.K., 2001. NMR and pattern recognition studies on the time-related metabolic effects of α -naphthylisothiocyanate on liver, urine, and plasma in the rat: An integrative metabonomic approach. *Chemical Research in Toxicology* 14, 1401–1412.
- Westerhuis, J.A., Hoefsloot, H.C.J., Smit, S., Vis, D.J., Smilde, A.K., van Velzen, E.J.J., van Duijnhoven, J.P.M., van Dorsten, F.A., 2008. Assessment of PLS-DA cross validation. *Metabolomics* 2008 (4), 81–89.
- Wu, H., Southam, A.D., Hines, A., Viant, M.R., 2008. High throughput tissue extraction protocol for NMR and mass spectrometry based metabolomics. *Analytical Biochemistry* 372, 204–212.
- Wu, H., Zhang, X., Li, X., Wu, Y., Pei, F., 2005. Acute biochemical effects of $\text{La}(\text{NO}_3)_3$ on liver and kidney tissues by magic-angle spinning ^1H nuclear magnetic resonance spectroscopy and pattern recognition. *Analytical Biochemistry* 339, 242–248.