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Benzo(*a*)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (^{1}H NMR) based metabolomics

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ABSTRACT

Benzo(*a*)pyrene is an important polycyclic aromatic hydrocarbon (PAH) which causes carcinogenic, teratogenic and mutagenic effects in various species and the level of contamination of this toxic agent in the marine environment is of great concern. In this study, metabolic responses induced by two doses (0.02 and 0.2 μM) of BaP were characterized in the gill tissues of Manila clam *Ruditapes philippinarum* after exposure for 24, 48 and 96 h. The high dose (0.2 μM) of BaP induced the disturbances in energy metabolism and osmotic regulation based on the metabolic biomarkers such as succinate, alanine, glucose, glycogen, branched chain amino acids, betaine, taurine, homarine, and dimethylamine in clam gills after 24 h of exposure. In addition, hormesis induced by BaP was found in clams exposed to both doses of BaP. Overall, our results demonstrated the applicability of metabolomics for the elucidation of toxicological effects of marine environmental contaminants in a selected bioindicator species such as the Manila clam.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have been ubiquitous contaminants in the environments due to anthropogenic activities, such as incomplete combustion of organic materials, petroleum spills, discharges from ships and industrial effluents (Latimer and Zheng, 2003; Phillips, 1983; Beyer et al., 2010; Cheng et al., 2010). PAH contamination is of great concern since these compounds are known to be cytotoxic, carcinogenic and mutagenic to various organisms (Uno et al., 2001; Toyooka and Ibuki, 2007; Salihoglu et al., 2010; Li et al., 2010). Benzo(*a*)pyrene (BaP) is an important and extensively studied member of PAHs (Zhang et al., 2004; Latimer and Zheng, 2003; Beyer et al., 2010). It can be metabolized to diverse exogenous metabolites including 3-hydroxybenzo(*a*)pyrene (3-OH BaP), 9-hydroxybenzo(*a*)pyrene (9-OH BaP), benzo(*a*)pyrene-4,5-dihydrodiol (BaP 4,5-diol), benzo(*a*)pyrene-7,8-dihydrodiol (BaP 7,8-diol), benzo(*a*)pyrene-9,10-dihydrodiol (BaP-9,10-diol) and benzo(*a*)pyrene quinones (BaP quinones) by mammalian and fish liver microsomes (Melius, 1984). The cytotoxic, carcinogenic and mutagenic effects of BaP in various species

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have been well-documented (Binelli et al., 2008; Poirier and Beland, 1992; Uno et al., 2001). For example, Poirier and Beland (1992) reported that the carcinogenicity of BaP was attributed to the reaction of BaP metabolites, primarily the diol epoxides, with DNA (Poirier and Beland, 1992). In China, the concentrations of PAHs are increasing in the marine and coastal environments (Zhang et al., 2004). Therefore it is necessary to assess the toxicological effects of PAHs in the marine organisms.

The traditional toxicological approaches focus on the measure of specific biological or biochemical responses (biomarkers), such as antioxidant enzymatic activities to test for oxidative stress and the expression of the protein vitellogenin to assess the endocrine disruption of contaminants (Hahlbeck et al., 2004; Hansen et al., 1998). Due to the recent developments in system biology (genomics, proteomics, transcriptomics and metabolomics), the approaches for toxicological biomarker discovery have been greatly expanded for the global biomarker discoveries (Daviss, 2005). Among these post-genomic techniques, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabolomics can perform a systematic study on the global metabolic profile, especially the low molecular-weight metabolites (<1000 Da) to characterize the impact on specific cellular processes (Daviss, 2005). Such study of metabolic profiling has been widely used in multiple research fields including drug toxicity, inborn disease diagnosis, functional genomics, and environmental toxicology (Brindle et al., 2002; Bundy et al., 2004; Wu et al., 2005; Viant et al., 2006a,b; Liu et al., 2011). Moreover, NMR spectroscopy is rapid and rich in structural and quantitative information and allows the metabolites to be analyzed simultaneously (Lindon et al., 2000; Wu et al., 2005). Therefore, the application of ^{1}H NMR spectroscopy in metabolomics can analyze a wide range of endogenous metabolites from biological samples including intact tissues or tissue extracts to provide valuable biochemical information on the physiological perturbations induced by both endogenous and exogenous factors (e.g., contaminants) (Fiehn, 2002). To our knowledge, however, the application of this metabolomics approach in marine toxicology of PAHs with invertebrates has been rather limited (Williams et al., 2009).

The Manila clam *Ruditapes philippinarum* is widely distributed along the coast of China and used as one of important sentinel organisms in 'Mussel Watch Program' launched in China in 2004. Since the clam filters large amounts of seawater due to their nutritional and respiratory needs, this species of mollusc can accumulate high levels of environmental contaminants. Due to the wide distribution, long life cycle, high tolerance to salinity and temperature, ease of collection and high bioaccumulation of contaminants, the Manila clam has been considered a good bioindicator in coastal sediment pollution assessments and environmental toxicology (Park et al., 2006, 2008; Ji et al., 2006; Liu et al., 2010).

The gill is a main target organ for the accumulation of contaminants in marine bivalve invertebrates (Panfoli et al., 2000). Hence, it is potentially sensitive and suitable for the detection of metabolic biomarkers induced by contaminants. In this limited study, therefore, ^{1}H NMR-based metabolomics was applied to gill tissue extracts from environmentally relevant concentrations of BaP-exposed Manila clam *R. philippinarum*, to characterize the metabolic responses and toxicological effects in gill tissues as to BaP after three (24, 48 and 96 h) exposure times.

2. Materials and methods

2.1. Experimental design

Adult Manila clams *R. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local unpolluted culturing farm (Bohai Sea, 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 3 flat-bottomed rectangular tanks with 20 L capacity and each tank contained 20 clams.

For the experiment, clams were treated with two nominal concentrations of BaP at 0.02 and 0.2 μM (originally dissolved in DMSO), respectively. These tested concentrations (0.02 and 0.2 μM) BaP have been found in some extremely polluted estuaries in China (Zhang et al., 2004) and proven to be toxic in a freshwater invertebrate, the zebra mussel *Dreissena polymorpha* (Binelli et al., 2008). Clams cultured in normal filtered seawater containing 0.002% DMSO (v/v) were used as control group. The clams ($n = 5$, replicates) were randomly sampled for the gill tissues 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 (~100 mg) was homogenized in 4 mL g^{-1} (solvent volume/tissue mass, the same as below) of methanol and 0.85 mL g^{-1} of water using a high throughput homogenizer, Precellys 24 (Bertin, France). The mixture was then shaken and centrifuged (5 min, 2000 × *g*, at 4 °C), and the supernatant 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 supernatant, and the mixture was vortexed and then 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 (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 *g* for 5 min at 4 °C. The supernatant (550 μL) was then 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) ^{1}H NMR spectra were obtained using a 11.9 μs pulse, 6009.6 Hz spectral width, mixing time 0.1 s, and 3.0 s relaxation delay with standard 1D NOESY pulse sequence, with 128 transients collected into 16, 384 data points. Datasets were 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 (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, ^{1}H–^{1}H 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 ^{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 generalized log (glog) 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 principal components analysis (PCA) was performed using PLS Toolbox software (version 4.0, Eigenvector Research, Manson, WA).

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 (Keun et al., 2003). 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. One way ANOVA (analysis of variance) 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 BaP-exposed groups. For the identification of significant metabolites, one way ANOVA was conducted on the ratio of representative bin area (peak intensity) of metabolites to the total spectral area as well. These significant metabolites were contributive for the separation between control and BaP-treated samples and hence were considered metabolic biomarkers induced by BaP exposures. A P value of 0.05 was considered significant for the multiple comparisons on the metabolites between control and exposed samples.

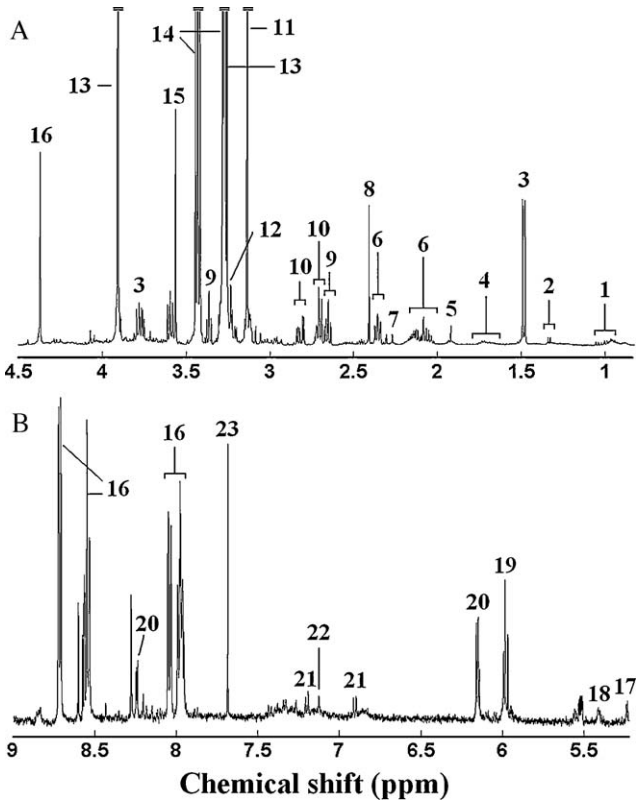

Fig. 1 – A representative one dimensional 500 MHz ^{1}H NMR spectrum of gill tissue extracts from a control clam (A) vertical expansion of the aromatic region (B). Keys: (1) branched chain amino acids: isoleucine, leucine and valine, (2) lactate, (3) alanine, (4) arginine, (5) acetate, (6) glutamate, (7) acetoacetate, (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) ATP/ADP, (21) tyrosine, (22) histidine and (23) unknown 2 (7.68 ppm).

3. Results

3.1. ^{1}H NMR spectroscopy of gill tissue extracts

Fig. 1 shows a representative 1-D ^{1}H NMR spectrum of gill tissue extracts from a control Manila clam (Fig. 1). The original NMR spectrum (Fig. 1A) is dominated by the organic osmolyte, betaine (3.25 and 3.91 ppm) and an organic acid, malonate (3.13 ppm), which are 10–100 times intense than other metabolites. However, the NMR intensities of scanty metabolites such as homarine and branched chain amino acids (valine, leucine and isoleucine) were apparently enhanced after glog-transformation (data not shown). Several classes of metabolites were assigned (Table 1), including amino acids (branched chain amino acids: valine, leucine, isoleucine, aspartate, glutamate, glycine, etc.), energy storage compounds (glucose, ATP/ADP and glycogen), organic osmolytes (betaine,

Table 1 – Chemical shifts for the hydrophilic metabolites obtained from 1D ^{1}H NMR spectra in 99.9% D_2O ($NaH_2PO_4 + Na_2HPO_4$ buffer, pH 7.0) and identified in Fig. 1.

Metabolite	Chemical shift (ppm)
Leucine	0.94 (t)
Isoleucine	0.98 (d)
Valine	1.05 (d)
Lactate	1.33 (d)
Alanine	1.48 (d)
Arginine	1.70 (m), 1.93 (m)
Acetate	1.92 (s)
Glutamate	2.10 (m), 2.35 (m), 3.75 (t)
Acetoacetate	2.27 (s)
Succinate	2.41 (s)
Aspartate	2.68 (ABX), 2.82 (ABX)
Malonate	3.13 (s)
Acetylcholine	3.21 (s)
Betaine	3.27 (s), 3.91 (s)
Taurine	3.25 (t), 3.43 (t)
Glycine	3.57 (s)
Homarine	4.37 (s), 7.98 (dd), 8.04 (d), 8.55 (dd), 8.72 (d)
α-Glucose	5.24 (d)
ATP/ADP	6.15 (d), 8.26 (s), 8.54 (s)
Tyrosine	6.88 (m), 7.20 (m)
Histidine	7.08 (s), 7.20 (d), 7.83 (s)

s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, ABX = complex multiplet involving 2 protons (A and B) and a heavy atom (X).

dimethylamine and homarine, etc.), and intermediates in tricarboxylic acid cycle (e.g., succinate) (Table 1, Fig. 1A and B).

3.2. *Principal components analysis on the ^{1}H NMR spectra of gill tissue extracts*

Principal components analysis was conducted on the NMR spectral data sets of gill tissue extracts after 24, 48 and 96 h exposures of BaP. After 24 h of exposure, the gill samples from control (inverted red triangles) and high dose (0.2 μM, blue squares) of BaP-exposed groups were significantly separated along PC1 ($P < 0.05$), while no separation between the control and low dose (0.02 μM, green cycles) of BaP-exposed groups were observed along either PC1 or PC2 axis (Fig. 2A). Interestingly, it was found that the low and high doses of BaP-exposed samples were significantly ($P < 0.05$) classified along negative and positive PC2 axes, respectively. The scores plot (PC1 vs. PC3) showed no clear separations between control and BaP-treated groups after exposure for 48 h. However, the low and high doses of BaP-treated groups were clearly ($P < 0.05$) separated along negative and positive PC3 axes, respectively (Fig. 3A). Similar to the PCA results after 48 h of exposure, the low and high doses of BaP-treated groups were significantly ($P < 0.05$) separated along negative and positive PC2 axes after 96 h of exposure, while no separations between the control and BaP-exposed groups were detected along any PC axis (Fig. 4A).

The plots (Figs. 2B, C, 3B and 4B) of PC loading were originally used to define the NMR spectral bins (peaks) of metabolites which might be significant for the separation between the control and dosed groups. Then, one way analysis of variance with a 5% significance level was applied to

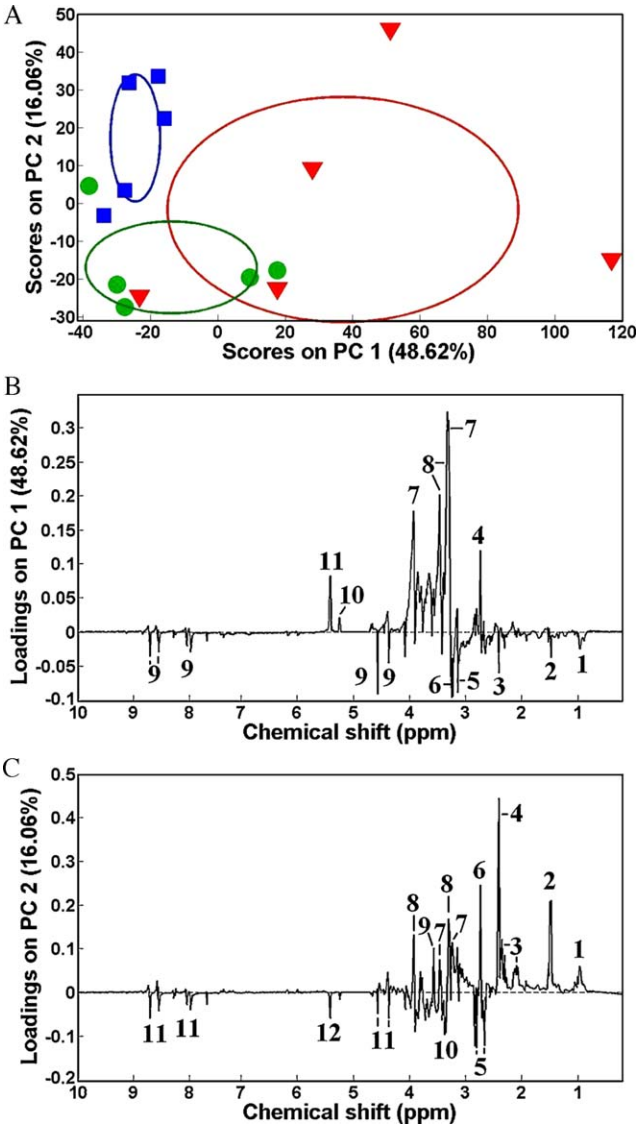

Fig. 2 – Principal components analysis (PCA) showing (A) separations (PC1 vs. PC2) between control (▼), 0.02 (●) and 0.2 μM (■) BaP-treated clam samples after exposure for 24 h, and corresponding PC1 (B) and PC2 (C) loadings plots showing the metabolic differences between the different groups of clam. 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) succinate, (4) dimethylamine, (5) malonate, (6) acetylcholine, (7) betaine, (8) taurine, (9) homarine, (10) α-glucose and (11) glycogen. Keys in (C): (1) branched chain amino acids: isoleucine, leucine and valine, (2) alanine, (3) glutamate, (4) succinate, (5) aspartate, (6) dimethylamine, (7) taurine, (8) betaine, (9) glycine, (10) hypotaurine, (11) homarine and (12) glycogen.

the NMR spectral bins that presented a possible contribution to the separation, facilitating the identification of metabolic changes.

Basically, the levels of branched chain amino acids (valine, leucine and isoleucine), alanine, succinate, malonate, phos-

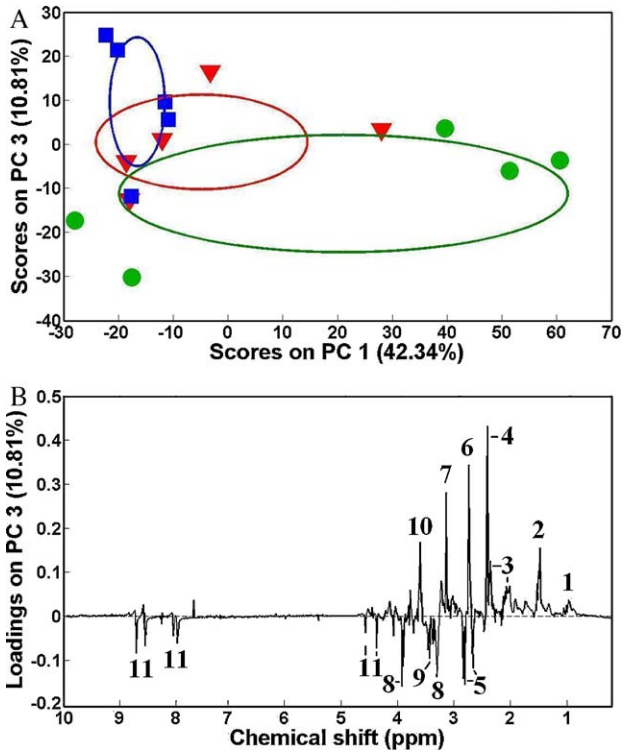

Fig. 3 – Principal components analysis (PCA) showing (A) separations (PC1 vs. PC3) between control (▼), 0.02 (●) and 0.2 μM (■) BaP-treated clam samples after exposure for 48 h, and corresponding PC3 (B) loadings plot showing the metabolic differences between the 0.02 and 0.2 μM BaP-treated clam samples. Ellipses represented mean ± SD of PC scores along both PC1 and PC3 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) taurine, (10) glycine and (11) homarine.

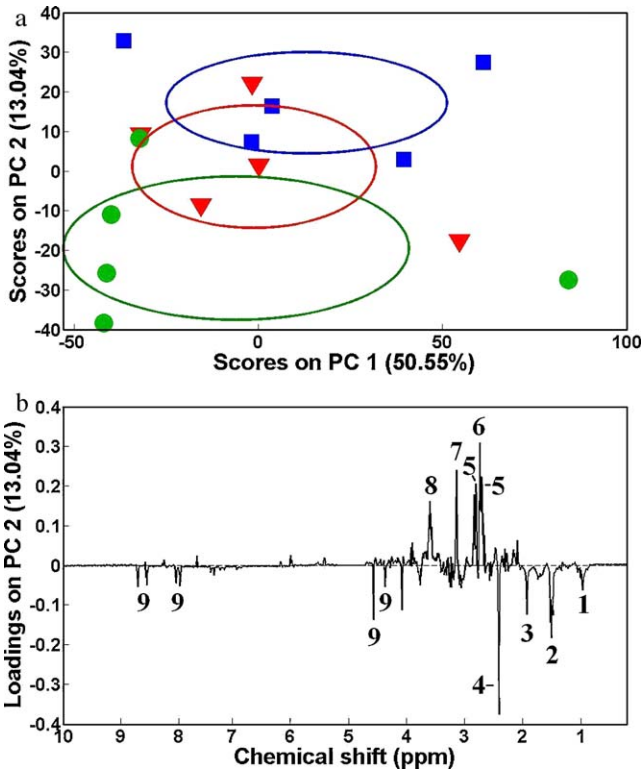

Fig. 4 – Principal components analysis (PCA) showing (A) separations (PC1 vs. PC2) between control (▼), 0.02 (●) and 0.2 μM (■) BaP-treated clam samples after exposure for 48 h, and corresponding PC2 (B) loadings plot showing the metabolic differences between the 0.02 and 0.2 μM BaP-treated clam samples. 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) aspartate, (6) dimethylamine, (7) malonate, (8) glycine and (9) homarine.

phocholine and homarine were significantly elevated in the gill tissues from the high (0.2 μM) dosed group after 24 h of exposure, while dimethylamine, betaine, taurine, glucose and glycogen were decreased significantly (Fig. 2B). For the significant separation between low (0.02 μM) and high doses (0.2 μM) of BaP exposures, the metabolic differences included relatively high levels of branched chain amino acids (valine, leucine and isoleucine), alanine, succinate, glutamate, dimethylamine, betaine, taurine and glycine in the high concentration (0.2 μM) of BaP-exposed clam samples, and high levels of aspartate, hypotaurine, homarine and glycogen in the low concentration (0.02 μM) of BaP-exposed clam samples (all significant at the 5% level) (Fig. 2C). After 48 h of BaP exposures, the significant metabolic differences in gill tissues between the low and high doses of BaP-exposed samples were the abundant branched chain amino acids (valine, leucine and isoleucine), alanine, glutamate, succinate, dimethylamine, malonate and glycine in the high dosed samples, and relatively high levels of aspartate, taurine, betaine and homarine in the low BaP-dosed group (Fig. 3B). The significant ($P < 0.05$) metabolic differences resulted in high abundances of dimethylamine, aspartate, malonate and glycine in high dose (0.2 μM) of BaP-exposed samples, together with the relatively high levels of branched chain amino acids, alanine, acetate, succinate and homarine in the low (0.02 μM) BaP-dosed group after 96 h of exposure (Fig. 4B).

4. Discussion

Metabolomics is a well-established and developing technique of system biology based on the global measurement and analysis of low molecular weight (<1000 Da) endogenous metabolites using modern analytical techniques such as NMR spectroscopy and Mass spectrometry (Plumb et al., 2003; Wang et al., 2003). The mechanisms of PAHs toxicity are very complex because PAHs can be metabolically activated by Phase I enzymes (e.g., cytochrome p450) to reactive intermediates that bind covalently to nucleic acids and proteins. Moreover, PAHs can induce numerous enzymes involved in activation and detoxification of PAHs via the aromatic hydrocarbon receptor (AHR), and PAHs can affect the expression of multiple other

genes by way of both AHR-dependent and AHR-independent mechanisms (Uno et al., 2001; Puga et al., 1992; Hankinson, 1995; Ryu et al., 1996). However, metabolomics can provide an overview on the toxicological effects of PAHs in organisms by characterizing the metabolic responses (biomarkers) without detailed knowledge of the complex biotransformation of PAHs (Lin et al., 2006; Stentiford et al., 2005).

After PCA analysis, the PC score plot (PC1 vs. PC2) showed clear separations along PC1 axis between the control and high concentration of BaP-dosed group (0.2 μM) after 24 h of exposure (Fig. 2A), which exhibited the metabolic responses induced by the high dose of BaP in clam gill samples. Although the clusters of control and BaP-exposed samples were not separated along PC2 axis, the high dose of BaP-treated group was classified upon positive PC2 axis from the low dose of BaP-treated group which was located along negative PC2 axis. It seemed there was a hormesis caused by various concentrations of BaP exposure. Hormesis is defined as the dose–response relationship phenomenon characterized by low-dose stimulation and high-dose inhibition, which has been frequently observed in toxicologically designed studies on the organic toxic chemicals (e.g., diethylstilbestrol and bisphenol A) (Vom Saal et al., 1997; Wetherill et al., 2002). Sverdrup et al. reported the hormesis phenomenon in the terrestrial plant, *Brassica alba* induced by BaP exposure (Sverdrup et al., 2007). In our case, we speculated that the 2 concentrations of BaP could induce the hormesis phenomenon in Manila clam *R. philippinarum*. However, a further study is needed to elucidate the mechanisms of BaP-induced hormesis in clam. After exposure to BaP for either 48 or 96 h, the low dose of BaP-exposed group with negative PC3 or PC2 scores was significantly ($P < 0.05$) separated from the high dose of BaP-exposed group which was located upon positive PC3 or PC2 axis (Figs. 3A and 4A). However, there was no significant classification between the control and BaP-exposed groups along any PC axis. It implied the recovery of metabolic response in the high dose of BaP-exposed clams after 48 and 96 h of exposures. In addition, the hormesis phenomena were also induced by these 2 doses of BaP in the clams.

The original NMR spectrum was dominated by an organic acid, malonate (3.13 ppm) and 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 intense than other metabolites. Malonate is a three-carbon dicarboxylic acid that can be found in diverse organism tissues including in the soybean tissues, rat brain, earthworm and mussel (Kim, 2002; Stumpf and Burris, 1981; Bundy et al., 2001). It is well known as a competitive inhibitor of cellular respiration since it binds to the active site of the succinate dehydrogenase in the tricarboxylic acid cycle. Organic osmolytes such as betaine, homarine, hypotaurine, dimethylamine, alanine (also involved in energy metabolism) 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 BaP, the disturbed metabolism in osmotic regulation was characterized by the altered osmolytes including the elevation of homarine and reduction of dimethylamine, betaine and taurine in the high dose (0.2 μM) of BaP-treated samples. The increased concentrations of branched chain amino acids were noticeable in the high dose (0.2 μM) of BaP-treated group. Viant et al. (2003) have reported that some marine molluscs used 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. Alanine is another important organic osmolyte like betaine, taurine, homarine and glycine in many invertebrates (Abe et al., 2005). It has also been reported that alanine 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). In some studies the increase in alanine caused by anoxia was correlated with an increase in succinate that is a clear biomarker of anaerobiosis in molluscs (De Zwaan et al., 1976). In this work, the high level of alanine and succinate clearly indicated the anaerobiosis in clam gills induced by 0.2 μM of BaP after 24 h of exposure. The decrease in glucose and glycogen was observed in the green mussel *Perna viridis* with cadmium and copper exposure for 1 week and suggested as the biomarkers of anaerobic metabolism in the previous study (Wu and Wang, 2010). Here the similar metabolic responses of depleted glucose and glycogen was the concomitant enhancement in anaerobic metabolism as well as the elevation of alanine and succinate in 0.2 μM of BaP-treated clam gills after 24 h of exposure. The precise biological role of malonate in clam is unknown, but it seems that the elevated malonate was correlated with the elevated succinate due to the inhibition of succinate dehydrogenase by malonate. However, the further study is needed to elucidate the detailed mechanisms.

The contrary metabolic differences between the low and high doses of BaP-exposed groups were observed by PCA analysis after exposure for 24, 48 and 96 h, which was likely caused by the hormesis of BaP in clam. As mentioned above, hormesis is a dose–responsive relationship phenomenon characterized by low-dose stimulation and high-dose inhibition of toxicants, which is generalizable as being independent of chemical agent (e.g., PAHs), biological model (e.g., vertebrates), and endpoint (e.g., enzymatic activities) measured (Edward et al., 2003). Hereby, the hormesis of BaP in clam could be detected by NMR-based metabolomics.

Based on the metabolic differences between 0.02 and 0.2 μM BaP-exposed groups, the major stimulation and inhibition effects were related to the osmotic regulation and energetic metabolism, which were characterized by the altered osmolytes (e.g., homarine, hypotaurine, betaine, taurine and amino acids) and energetic-related metabolites (e.g., succinate, alanine and glycogen). Since the original experimental design was to assess the toxicological effects of BaP at two environmental relevant concentrations which have been found in some estuaries of China (Zhang et al., 2004), a subtle study is needed to illustrate the hormesis of BaP in clam.

5. Conclusions

This work examined the metabolic responses and toxicological effects in the gill tissues of Manila clam *R. philippinarum* induced by two doses (0.02 and 0.2 μM) of BaP after exposure for 24, 48 and 96 h. The high dose (0.2 μM) of BaP induced the disturbances in energy metabolism and osmotic regulation due to the metabolic biomarkers such as succinate, alanine, glucose, glycogen, branched chain amino acids, betaine, taurine, homarine, and dimethylamine in clam gills after 24 h of exposure. In addition, the hormesis induced by BaP was found in clam exposed to these two doses of BaP. Overall, our results demonstrated the applicability of metabolomics for the elucidation of toxicological effects of marine environmental contaminants in the bioindicator.

Conflict of interest

Nothing declared.

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