Dose-dependent effects induced by cadmium in polychaete *Perinereis aibuhitensis*

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ABSTRACT

Cadmium is a known metal contaminant in the Bohai Sea. In this study, the dose-dependent responses induced by Cd were characterized in marine polychaete *Perinereis aibuhitensis* using the endpoints, including activities of enzymes, expression levels of stress-responsive genes and metabolic responses. Both enzyme activities and gene expression levels exhibited the hormetic effects induced by Cd in *P. aibuhitensis*, as shown by the typical U-shaped or inverted U-shaped response profiles. The highest concentration (1280 μg/L) of Cd exposure induced obvious oxidative stresses. NMR-based metabolomics revealed that Cd induced both linear dose-dependent effects (69.13% of the total variation) and a relatively slight hormesis (5.54% of the total variation) in energy metabolism in *P. aibuhitensis* at metabolite level. In details, Cd exposures linearly reduced the consumption of amino acids and enhanced the consumption of glucose for energy supply, resulting in elevated contents of amino acids and depleted contents of glucose. Additionally, Cd treatments induced hormesis in the conversion of ATP hydrolysis to AMP. This work suggested that the hormetic effects should be considered in the ecological risk assessment for the environmental pollutants.

1. Introduction

Cadmium (Cd) is an important industrial material. It is also an impurity with some minerals, such as zinc (Zn). Therefore, Cd can easily enter into the environment. As a nonessential metal element, Cd can be strongly bio-accumulated and its excessive level can induce diverse toxicities in organisms (Bertin and Averbeck, 2006; De Silva et al., 2018; Pavlakia et al., 2017). Along the Bohai coast, there are numerous metal smelteries with unavoidable pollution discharges containing Cd. Many surveys have reported the Cd pollution in the Bohai Sea (Gao et al., 2014; Ji et al., 2016). Our studies have also found that Cd is one of the most frequently detectable metal contaminants in marine organisms sampled from the Bohai Sea (Ji et al., 2016; Xu et al., 2016). In 2016, the significant Cd accumulations were detected in shrimps *Crangon affinis* sampled from several sites along the Laizhou Bay in the Bohai Sea (Xu et al., 2016). In the gastropods and oysters collected in 2002 along the coastline of the Bohai Sea, the concentration of Cd significantly exceeded the maximum permissible level (MPL) for Cd in food product recommended by the World Health Organization (WHO)

(Liang et al., 2004). The seawater concentration of Cd reached 5 μg/L that was detected in the seawater from the Jinzhou Bay in the Bohai Sea from 1987 to 1990 (Ma et al., 1995). The maximum seawater concentration of Cd was approximately 16 μg/L detected in the seawater from the Liaodong Bay in the Bohai Sea in 2000 (Zhang, 2001). These evidence confirmed the historic contamination of Cd in the Bohai Sea, which may pose threats on the marine organisms and human body via food chain transfer. Therefore, it is necessary to investigate the toxicological effects of Cd in marine organisms.

Usually, the environmental concentration of Cd in seawater is as low as the level of μg/L. In toxicology, however, there is an interesting dose-response relationship named hormesis when the toxicant concentration is very low (Chapman, 2002). The hormesis is generally characterized by the low-dose stimulation and high-dose inhibition phenomenon that significantly influences the ecological risk assessment for environmental pollutants. Based on its definition, the hormesis refers to a biphasic but a linear dose response to the toxicants (Calabrese and Baldwin, 2000). The hormetic effect was hypothesized to be the overcompensation to a perturbation in homeostasis (Stebbing, 1998,

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2000). In other words, organisms can not only repair the damage, but also more effectively overcompensate and reduce background damage induced by stressors, such as pollutants. This hormetic effect can be characterized by either a U-shaped or an inverted U-shaped dose response profile, depending on the endpoints measured (Calabrese and Baldwin, 2003). In ecotoxicology, the hormetic effects of Cd have been frequently observed. Zhang et al. (2009) found that the low concentrations of Cd increased the activities of catalase (CAT) and superoxide dismutase (SOD), but high concentrations of Cd inhibited the activities of these enzymes in an inverted U-shaped curve in earthworm *Eisenia fetida*. In another study, Nascarella et al. (2003) observed that low levels of cadmium significantly enhanced the pupation rate, while higher doses inhibited pupation rate of larval queen blowfly *Phormia regina*, resulting in an inverted U-shaped response profile. In marine ecotoxicology, however, there are few reports related to Cd-induced dose-dependent responses in marine organisms.

The marine polychaete *Perinereis aibuhitensis* is a key species of the benthic community in coastal sediments. Since this sediment-dwelling ragworm is sensitive to marine pollutants, including metals (Pb, Hg, etc.) and organic compounds (petroleum hydrocarbons), it has been increasingly used as a bioindicator in marine ecotoxicology recently (Tian et al., 2014; Zhang et al., 2017). In this work, the marine polychaete *P. aibuhitensis* was used to test the dose-dependent responses of Cd. The endpoints, including activities of enzymes, expression levels of stress-responsive genes and metabolite profiles, were characterized to elucidate the dose-dependent responses of Cd in the marine polychaete *P. aibuhitensis*.

2. Materials and methods

2.1. Experimental animals and conditions

One hundred and twenty adult individuals of *P. aibuhitensis* (weight: 1.9 ± 0.3 g) were collected from a culturing farm in Dongying, China. After transported to the culture laboratory, the animals were allowed to acclimate in aerated seawater (20 ± 0.5 °C, 32.5 psu, collected from a pristine environment) in the laboratory for 1 week. After acclimation, the individuals of *P. aibuhitensis* were randomly divided into six groups (one control and five Cd exposures, respectively). Each group contained two replicate beakers each containing ten individuals of *P. aibuhitensis* in 3 L of aerated seawater. Five sublethal concentrations (5, 20, 80, 320 and 1280 μg/L) of Cd were used for the dose-dependent exposures for *P. aibuhitensis*. The highest concentration (1280 μg/L) of Cd was approximately 10% of the LC50 ($\sim$12,000 μg/L, unpublished value in our laboratory) for *P. aibuhitensi* at 96 h. After exposure for 96 h, 10 individuals of *P. aibuhitensis* were randomly sampled from each group. During acclimation and exposure periods, these animals were fasted and no mortality was found. Each sample was snap-frozen in liquid nitrogen and ground into a homogeneous mixture under liquid nitrogen. Then the ground mixture was divided into four parts for metabolite extraction, RNA extraction, enzyme activity measurement and Cd determination. These samples were stored at −80 °C before further procedures.

2.2. Metabolite extraction

Polar metabolites in the whole soft tissues of *P. aibuhitensis* ($n = 10$ for each treatment) were extracted by the modified metabolite extraction protocol as described previously (Zhang et al., 2011). Briefly, the soft tissue (ca. 100 mg wet weight) was homogenized and extracted using a solvent combination of 4 mL/g of methanol, 5.25 mL/g of water and 2 mL/g of chloroform. The polar layer (methanol/water) with metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts were then dissolved in 600 μL of phosphate buffer (100 mM Na_2HPO_4 and NaH_2PO_4, containing 0.5 mM TSP as the internal standard, pH 7.0) in D_2O. The mixture was vortexed and

centrifuged at 3000g for 5 min at 4 °C. A volume of 550 μL of the supernatant substance was pipetted into a 5 mm NMR tube for NMR analysis.

2.3. ^{1}H NMR spectroscopy

Metabolite extracts of *P. aibuhitensis* were analyzed on a Bruker AV 500 NMR spectrometer at 25 °C as described previously (Zhang et al., 2011). All ^{1}H NMR spectra were manually phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) using TopSpin (version 2.1, Bruker).

2.4. Spectral pre-processing and data analysis

All one dimensional ^{1}H NMR spectra were converted to a data matrix using the ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA) (Viant et al., 2009). Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were removed from the NMR spectral data matrix. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. Principal components analysis (PCA) was performed to detect the dose-dependent response profiles related to Cd exposures using PLS Toolbox (version 4.0, Eigenvector Research, Manson, WA) (Zhang et al., 2011). Metabolites were identified and quantified by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada) (Viant et al., 2009). The metabolite concentrations (mM) were determined by the concentration (0.5 mM) of internal standard, TSP.

2.5. Enzyme activities

The enzyme activities in *P. aibuhitensis* ($n = 10$) were measured using a multimode microplate reader (Infinite M200, TECAN, Switzerland) according to the manufacturer's protocols for enzyme kits (Jiancheng, Nanjing, China). In this work, the enzymes for the activity measurement included superoxide dismutase (SOD, EC 1.15.1.1), glutathione S-transferases (GST, EC 2.5.1.18), catalase (CAT, EC 1.11.1.6) and acetylcholinesterase (AChE, EC 3.1.1.7). Protein concentration was determined using the Coomassie brilliant blue G-250 dye-binding method with bovine serum albumin as standard (Bradford, 1976). The unit of enzymes was defined as the activity of an enzyme per milligram of total protein (U/mg protein).

2.6. RNA extraction and quantitation of gene expressions

Total RNA from the soft tissue samples of *P. aibuhitensis* ($n = 10$) was extracted following the manufacturer's directions (Invitrogen), and the first strand cDNA was synthesized according to M-MLV RT Usage information (Promega). For gene expression analysis, eight stress-responsive genes were selected and the corresponding primers were listed in Table 1. These genes for expression quantification included *SOD*, *CAT*, heat shock protein 70 (*HSP 70*), heat shock protein 90 (*HSP 90*), cytochrome P450 (*CYP450*), cytochrome oxidase subunit I (*COI*), metalloprotein (*MP*) and vitellogenin (*VTG*). The beta-actin gene (*β-actin*) was chosen as the reference gene and its primers were also listed in Table 1.

The fluorescent real-time quantitative PCR amplifications were conducted in triplicate in a total volume of 20 μL, containing 10 μL of $2 \times$ SYBR Premix Ex Taq™ (TaKaRa), 0.4 μL of $50 \times$ ROX Reference DYE II, 4.8 μL of DEPC-treated H_2O, 0.4 μL of each primer and 4.0 μL of 1:20 diluted cDNA. The PCR program was as following: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 45 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the ABI 7500 SDS software (Applied Biosystems). To maintain

Table 1
The list of primers used for the determination of internal control and quantification of gene expressions by qPCR.

Gene name	Accession No.	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-actin	JN609588	GGGCTACTCCTTCACCACCA	CGAAGTCCAGAGCAACATAGCA
SOD	KF199864	TGCTGATGTAGATGACCTTG	CTATTGAGTGATTCCGATGAC
CAT	KF199863	GCCCAGCCCACTTCATTC	CTCCCTCTTGGTTATCAGACAC
HSP 70	HQ449186	ACGCTACACACGAGAAAACACAA	GAAAACCCAACGCAGGA
HSP 90	KU535887	CTACTCAGCCTATCTTGTG	CCTTGATTCTCTTCTCTTCC
CYP 450	HM126463	ACACCACAGCATCAGGAATATC	CGCCAGCAACTCGTCAAG
COI	GU362686	CGACAGTCATCAACATACGCTCAA	TCCTCCCGCAGGGTCAAAG
MP	FJ212325	CGTCAACCACATCAAGGGAAC	CCCGCTGAAACGACACTAACA
VTG	KF212194	CCGAGAAGGTTGGAGATGTG	GGAGGATGTAGTCAGCAATGG

Abbreviations: *SOD*, superoxide dismutase; *CAT*, catalase; *HSP 70*: heat shock protein 70; *HSP 90*: heat shock protein 90; *CYP 450*: cytochrome P450 family 4 protein; *COI*: cytochrome oxidase subunit I; *MP*: metalloprotein; *VTG*: vitellogenin.

consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was employed to analyze the expression levels of these genes (Livak and Schmittgen, 2001).

2.7. Cd determination

The soft tissue samples ($n = 10$) of *P. aibuhitensis* were dried at 80 °C to constant weights. The dried tissues were digested in concentrated HNO_3 (70%, Fisher Scientific) in a microwave digestion system (CEM, MAR5). The samples were heated in a microwave oven (heating to 200 °C and holding at 200 °C for 15 min). All digested samples were diluted with ultrapure water for Cd determination using ICP-MS technique (Agilent 7500i, Agilent Technologies Co. Ltd., Santa Clara, CA, USA). GBW08571 Marine mussel tissue was used as a certified reference for Cd determination to ensure internal quality assurance/quality control (QA/QC) practices (Li et al., 2012). The recovery was restricted within 95.5–104.3% for Cd.

2.8. Statistical analysis

The biochemical indices, including Cd concentrations, enzyme activities, metabolites and gene expressions in the samples of *P. aibuhitensis*, were expressed as means ± standard deviation and tested for normal distribution (Ryan-Joiner's test) and homogeneity of variances (Bartlett's test). One way analysis of variance (ANOVA) with Tukey's test was conducted on the biochemical indices from control and Cd treatments, respectively. The *P* values less than 0.05 were considered statistically significant. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.

3. Results and discussion

### 3.1. Cd accumulation in P. aibuhitensis

Cd can be easily bio-accumulated and induce toxic effects in organisms (Bertin and Averbeck, 2006; Gu et al., 2018). Although the average tissue Cd concentration was higher in the lowest concentration (5 μg/L) of Cd-exposed group than that in control group after Cd exposure for 96 h, there was no significance between these two groups because of the short exposure time (Fig. 1). For other four Cd-treated groups, the *P. aibuhitensis* accumulated significant ($P < 0.05$ or 0.01) amounts of Cd in the soft tissues. Apparently, the accumulated Cd contents in Cd treatments were significantly ($P < 0.05$) increased with the gradually increased Cd concentrations for exposures (Fig. 1). Especially, Cd accumulations in the two highest concentrations (320 and 1280 μg/L) of Cd treatments were approximately 20 and 60 times higher than that in controls, respectively. The significant accumulations of Cd might induce detectable toxicological effects in *P. aibuhitensis*.
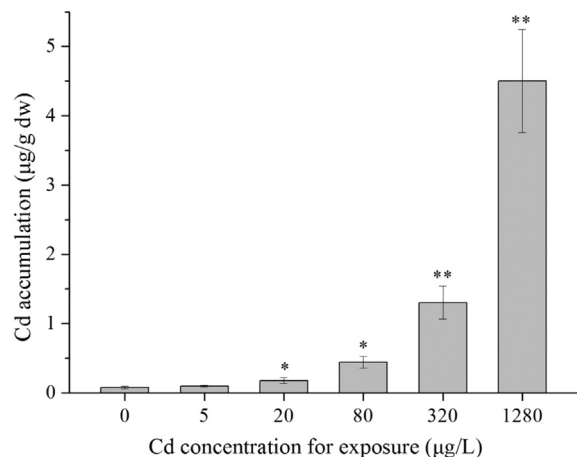
Fig. 1. The accumulated Cd concentrations (μg/g dry weight) in polychaete *P. aibuhitensis* after Cd exposures for 96 h. Cd concentrations are presented as the mean ± standard deviation. Statistical significances ($P < 0.05$, * and $P < 0.01$, **) between control and Cd treatments were determined by one way ANOVA.

3.2. Dose-dependent effects of Cd on the enzyme activities in P. aibuhitensis

To explore the dose-dependent effects of Cd in *P. aibuhitensis*, several biochemical endpoints, including enzyme activities, gene expression levels and metabolites, were tested. As shown in Fig. 2, the responses of SOD activities in *P. aibuhitensis* presented an inverted U-shaped curve, while the responses of CAT activities interestingly revealed a typical U-shaped curve. This finding clearly demonstrated the hormetic effects of Cd in *P. aibuhitensis*. Zhang et al. also observed the hormetic effects of Cd in earthworm *Eisenia fetida*, using SOD and CAT activities as the endpoints (Zhang et al., 2009). At cellular level, the exogenous toxicants often induce oxidative stress in organisms, resulting from the excessive production of reactive oxygen species (ROS). SOD is an enzyme that catalyzes the dismutation of the superoxide ($O_2{}^{-}$) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). CAT can catalyze the decomposition of hydrogen peroxide to water and oxygen. These two enzymes are very important enzymes in protecting the cell from oxidative damage by ROS. Cd is a known toxicant that induces oxidative stress in organisms (Bertin and Averbeck, 2006). In this work, the elevated SOD activities confirmed that Cd produced excessive superoxide radicals in *P. aibuhitensis*. In the highest concentration (1280 μg/L) of Cd treatment, the SOD activity somewhat recovered (Fig. 2). However, the CAT activity was highly significantly ($P < 0.01$) increased. It seemed that the highest concentration (1280 μg/L) of Cd induced more hydrogen peroxide but superoxide in *P. aibuhitensis*. GST, as the phase II metabolic isozyme, is known to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. AChE
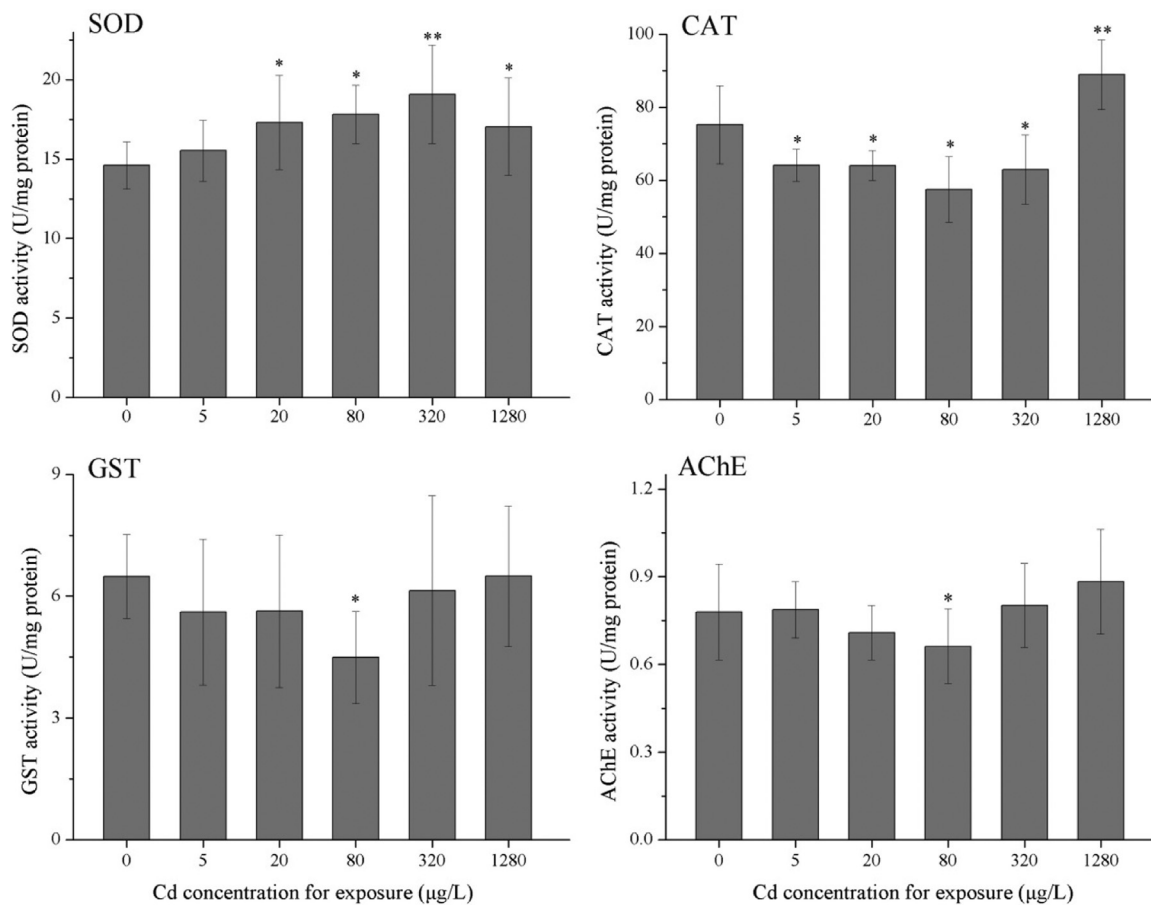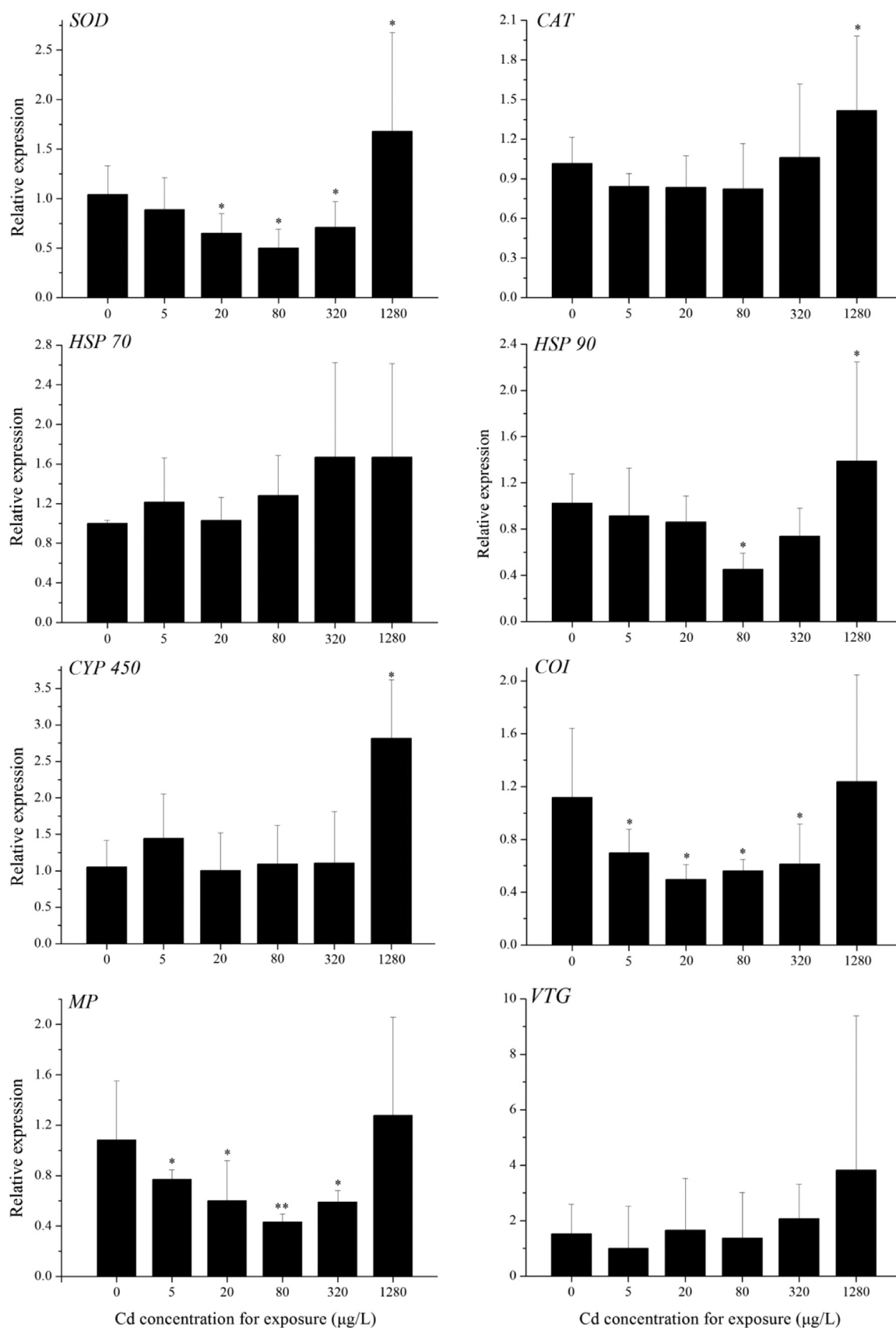
Fig. 2. The activities (U/mg protein) of SOD, CAT, GST and AChE in the tissues from *P. aibuhitensis* after Cd exposures for 96 h. Statistical significances ($P < 0.05$, * and $P < 0.01$, **) between control and Cd treatments were determined by one way ANOVA. **Abbreviations:** SOD, superoxide dismutase; CAT, catalase; GST, glutathione S-transferases; AChE, acetylcholinesterase.

catalyzes the breakdown of acetylcholine and of other choline esters that function as neurotransmitters (Quinn, 1987). It is therefore a known biomarker of neurotoxicity. For both GST and AChE, their activities were only significantly inhibited by the moderate concentration (80 μg/L) of Cd in *P. aibuhitensis*. Other concentrations of Cd for exposure didn't induce significant changes in GST and AChE activities. However, their dose response profiles were displayed in U-shaped-like curves, which suggested the slight hormetic effects of Cd in *P. aibuhitensis*, using GST and AChE activities as the endpoints.

### 3.3. Dose-dependent effects of Cd on the gene expression levels in P. aibuhitensis

Few researches focused on the hormetic effects of Cd or other toxicants in marine ecotoxicology at mRNA level. In this work, eight typical stress-responsive genes, including *SOD*, *CAT*, *HSP 70*, *HSP 90*, *CYP450*, *COI*, *MP* and *VTG*, were selected for the quantification of mRNA expressions. Among these genes, *SOD*, *CAT*, *HSP 70*, *HSP 90*, *CYP450* and *COI* are invariably associated with oxidative stress induced by the increased reactive oxygen species production and cellular toxicity caused by toxicants (Fridovich, 1995; Lewis et al., 1999; Srinivasan and Avadhani, 2012). The metalloprotein (*MP*) has a capacity to bind metals resulting in detoxification of metals, such as Cd (Yang et al., 2011). Vitellogenin (*VTG*) is a precursor protein of egg yolk used as a biomarker of reproductive toxicity to environmental toxicants which stimulate elevated levels of vitellogenin in organisms (Liu et al., 2016). After exposure with Cd for 96 h, the expression profile of *SOD* indicated a U-shaped curve (Fig. 3) that is interestingly opposite to that presented by SOD activities (Fig. 2). The disparity between mRNA expression of

SOD and corresponding SOD activity was not surprising, since mRNA expression means the tendency of its encoded protein. However, it does not always happen because of the posttranscriptional and posttranslational modifications and differential degradation rates between mRNA and protein (enzyme). In addition, the activities of SOD include the total activities of the SOD family. For both *CAT* and *HSP 90*, the expression profiles of their mRNAs were shown in U-shaped-like curves with or without significances for the expression levels from each Cd treatment (Fig. 3), which confirmed the hormetic effects induced by Cd in *P. aibuhitensis* at mRNA level, together with the expression profiles of *SOD* and *MP*. In details, the gene expressions of *SOD*, *CAT*, *HSP 90* and *MP* were more obviously inhibited in the moderate concentration (80 μg/L) of Cd-treated group, while the most significant ($P < 0.05$) overexpressions of these genes (except *MP*, $P > 0.05$) were observed in the highest concentration (1280 μg/L) of Cd-treated group. It suggested that the highest concentration of Cd induced clear oxidative stress and MP was mobilized to detoxify the toxicity of excessively accumulated Cd in *P. aibuhitensis*. The expression profile of *COI* was also seen in a U-shaped curve demonstrating the hormesis of Cd in *P. aibuhitensis*. However, the most significant inhibition of *COI* in *P. aibuhitensis* was caused by the second lowest concentration (20 μg/L) of Cd but the moderate concentration (80 μg/). For *HSP 70*, *CYP 450* and *VTG*, their mRNA expression profiles were found in neither linear responses nor hormetic curves. It might be interpreted that the hormesis for these genes could be located at other Cd concentration ranges. To achieve an overall view on the dose-dependent responses at mRNA level in *P. aibuhitensis* to Cd, a global analysis of gene expression (transcriptomics) is necessary in the future work.

Fig. 3. Expression levels of *SOD*, *CAT*, *HSP 70*, *HSP 90*, *CYP 450*, *COI*, *MP* and VTG relative to *β-actin* measured by qPCR in the tissues from *P. aibuhitensis* after Cd exposures for 96 h. Statistical significances ($P < 0.05$, * and $P < 0.01$, **) between control and Cd treatments were determined by one way ANOVA. **Abbreviations:** *SOD*, superoxide dismutase; *CAT*, catalase; *HSP 70*: heat shock protein 70; *HSP 90*: heat shock protein 90; *CYP 450*: cytochrome P450 family 4 protein; *COI*: cytochrome oxidase subunit I; *MP*: metalloprotein; *VTG*: vitellogenin.
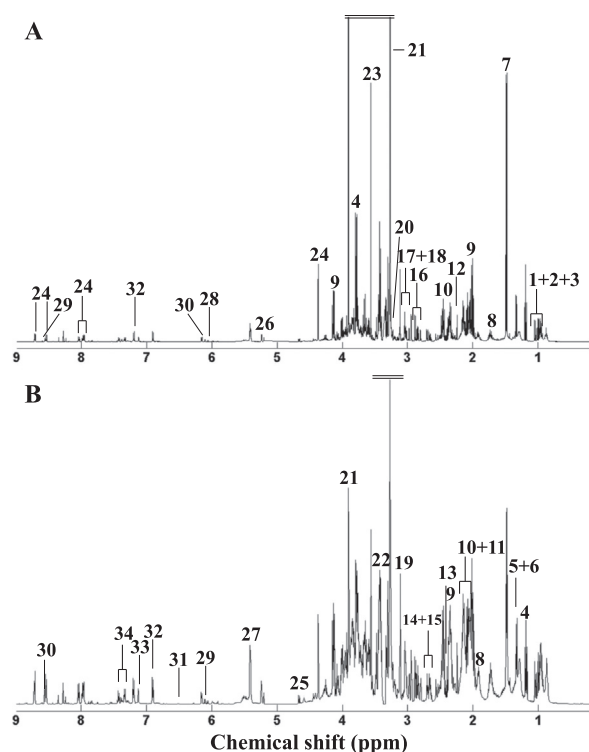
Fig. 4. A representative 1-dimensional ^{1}HNMR spectrum of metabolite extracts of *P. aibuhitensis* from control group, in original (A) and generalized log-transformed (B) forms. **Keys:** (1) isoleucine, (2) leucine, (3) valine, (4) ethanol, (5) lactate, (6) threonine, (7) alanine, (8) saccharopine, (9) proline, (10) glutamate, (11) glutamine, (12) acetone, (13) succinate, (14) hypotaurine, (15) aspartate, (16) asparagine, (17) creatine phosphate, (18) creatine, (19) malonate, (20) phosphocholine, (21) betaine, (22) taurine, (23) glycine, (24) homarine, (25) β-glucose, (26) α-glucose, (27) glycogen, (28) inosine, (29) AMP, (30) ATP, (31) fumarate, (32) tyrosine, (33) histidine and (34) phenylalanine.

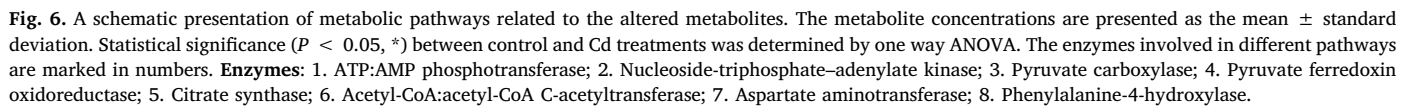### 3.4. Dose-dependent effects of Cd at metabolite level in P. aibuhitensis

Metabolomics has been widely used in ecotoxicology (Viant et al., 2009). In this work, NMR-based metabolomics was applied to detect the dose-dependent effects of Cd in *P. aibuhitensis* at metabolite level. A total of 34 metabolites in the whole tissue extracts of *P. aibuhitensis* were identified and labeled in Fig. 4. These metabolites were classified into several classes, including amino acids (isoleucine, leucine, valine, alanine, glycine, etc.), intermediates in the Krebs cycle (succinate and fumarate), organic osmolytes (hypotaurine, betaine, taurine and homarine) and energy storage compounds (glucose, ATP and glycogen). Principal component analysis (PCA) was performed on the NMR spectral data from control and all concentrations of Cd-treated groups. As shown in Fig. 5A and B, the average PC scores along PC1 (38.39% variation) and PC2 (30.74% variation) axes were linearly decreased and increased, respectively. The two higher concentrations (320 and 1280 μg/L) of Cd-treated groups were significantly ($P < 0.05$) separated along PC1 axis. Except the lowest concentration (5 μg/L) of Cd-treated group, the other four Cd-treated groups were significantly ($P < 0.05$) discrete along PC2 axis. At metabolite level, obviously, Cd mainly induced a linear dose-dependent response profile, which accounted for 69.13% of the variation induced by Cd in *P. aibuhitensis*. Interestingly, the average PC3 scores for all the six groups (control and five Cd treatments) were shown in a U-shaped curve with the moderate concentration (80 μg/L) of Cd treatment located at the bottom (Fig. 5C), which was basically consistent with the profiles from CAT, GST and AChE activities and *SOD*, *HSP 90* and *MP* expression levels. These findings confirmed the hormesis of Cd to *P. aibuhitensis* at metabolite level. However, PC3 could only interpret 5.54% of the variation
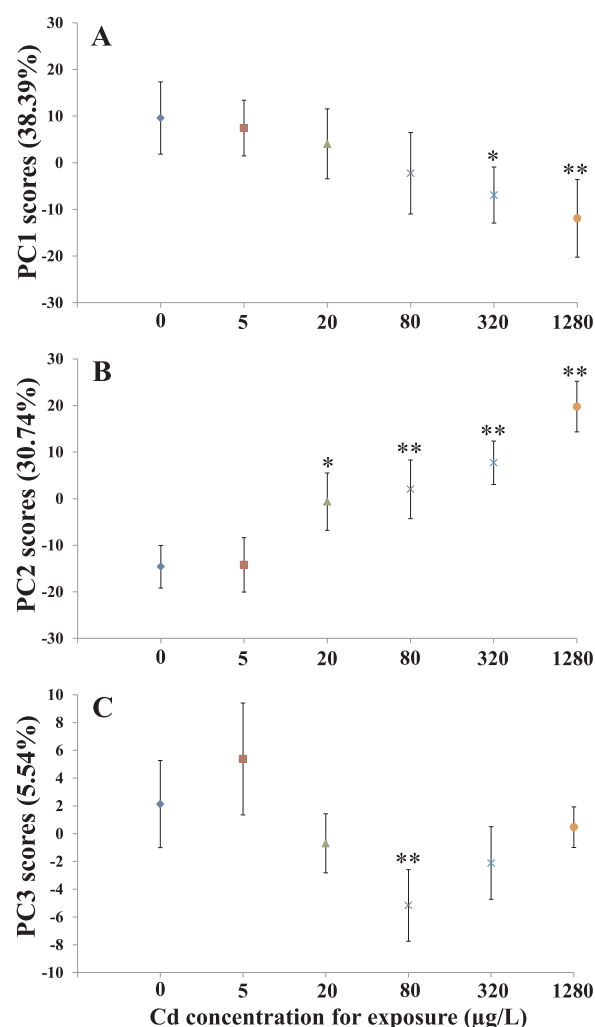
Fig. 5. The average PC scores of control and Cd-treated groups along PC1 (A), PC2 (B) and PC3 (C) axes. PC scores are presented as the mean ± standard deviation for each group. Statistical significances ($P < 0.05$, * and $P < 0.01$, **) between control and Cd treatments were determined by one way ANOVA.

induced by Cd. Therefore, Cd mainly induced linear dose-dependent effects and a relatively slight hormesis in *P. aibuhitensis* at metabolite level.

To characterize the dose-dependent responses to Cd exposures at metabolite level, all the metabolite concentrations from control and Cd treatments were quantified using the Chenomx software. One way analysis of variance (ANOVA) with Tukey's test indicated that these metabolites, including branched chain amino acids (BCAAs), saccharopine, glutamine, proline, aspartate, inosine, tyrosine, phenylalanine, acetone, glucose, homarine, AMP and ATP, were significantly altered in Cd-treated groups. The significantly altered metabolites in response to Cd exposures were summarized in the schematic metabolic pathways (Fig. 6). Basically, most of the responsive metabolites, including leucine, isoleucine, valine, saccharopine, tyrosine, phenylalanine, glucose and aspartate, were shown in linear dose-dependent profiles, which was consistent with the most variation (69.13%) interpreted by the linear dose-dependent profiles resulted in both PC1 and PC2 scores, as mentioned above. In marine invertebrates, amino acids are not only involved in energy metabolism, but also used as osmolytes to balance the osmolarity with the saline environment (Preston, 2005; Viant et al., 2003). In this work, the concentrations of amino acids (leucine, isoleucine, valine, tyrosine, phenylalanine and aspartate) were increased with the increased Cd concentrations for exposures. Interestingly, the

Fig. 6. A schematic presentation of metabolic pathways related to the altered metabolites. The metabolite concentrations are presented as the mean ± standard deviation. Statistical significance ($P < 0.05$, *) between control and Cd treatments was determined by one way ANOVA. The enzymes involved in different pathways are marked in numbers. **Enzymes**: 1. ATP:AMP phosphotransferase; 2. Nucleoside-triphosphate–adenylate kinase; 3. Pyruvate carboxylase; 4. Pyruvate ferredoxin oxidoreductase; 5. Citrate synthase; 6. Acetyl-CoA:acetyl-CoA C-acetyltransferase; 7. Aspartate aminotransferase; 8. Phenylalanine-4-hydroxylase.

concentrations of glucose were decreased with the increased Cd concentrations for exposures. The contents of these amino acids were roughly negatively correlated with the contents of glucose in Cd-treated *P. aibuhitensis*, which suggested that Cd exposures reduced the consumption of amino acids and enhanced the consumption of glucose for energy supply. Therefore, these altered amino acids were mainly involved in energy metabolism, as shown in the metabolic pathways in Fig. 6. Since saccharopine is an intermediate in the degradation of lysine, it might be also involved in energy metabolism (Pena et al., 2017).

Among these metabolites with significances, ATP and AMP were altered in U-shaped and inverted U-shaped profiles, respectively. Obviously, Cd treatments induced hormesis in the conversion of ATP hydrolysis to AMP. Acetone, a metabolite involved energy metabolism at well, was significantly decreased in the lowest concentration (5 μg/L) of Cd-treated group, while it was significantly increased in the moderate concentration (80 μg/L) of Cd-treated group. This also demonstrated the hormesis of Cd in *P. aibuhitensis*. However, the Cd concentration range of hormesis was located from 5 to 80 μg/L using acetone as the endpoint. Other metabolites, such as glutamine, proline and inosine, were elevated in some of the Cd treatments without obvious dose-dependent tendencies and probably related to disturbed energy metabolism. As an organic osmolyte, homarine was uniquely decreased in the second lowest concentration (20 μg/L) of Cd-treated group, which suggested the disturbance in the osmoregulation caused by this Cd exposure. Inosine is a nucleotide that is involved in a chain of

purine nucleotides reactions required for muscle movements. It was significantly increased in the moderate (80 μg/L) and highest (1280 μg/L) concentrations of Cd treatments, suggesting the perturbation in muscle movements induced by these two Cd exposures.

In conclusion, this study revealed the dose-dependent responses induced by Cd in marine polychaete *P. aibuhitensis*. The endpoints, including activities of enzymes, expression levels of stress-responsive genes and metabolic responses, were characterized to elucidate the dose-dependent responses of Cd in *P. aibuhitensis*. Both enzyme activities and gene expression levels exhibited the hormetic effects induced by Cd in *P. aibuhitensis*, resulting in the observable U-shaped or inverted U-shaped response profiles. At metabolite level, however, Cd mainly induced linear dose-dependent effects (69.13% of the total variation) and a relatively slight hormesis (5.54% of the total variation) in *P. aibuhitensis*. Overall, the moderate (80 μg/L) or the second lowest (20 μg/L) concentration and the highest concentration (1280 μg/L) of Cd exposures presented typical hormetic responses at enzyme activity, gene expression and metabolite levels. The highest concentration (1280 μg/L) of Cd exposure induced obvious oxidative stress. The metabolic responses suggested that Cd treatments induced both linear and hormetic effects in energy metabolism. In details, Cd exposures linearly reduced the consumption of amino acids and enhanced the consumption of glucose for energy supply, resulting in elevated contents of amino acids and depleted contents of glucose. Additionally, Cd treatments induced hormesis in the conversion of ATP hydrolysis to AMP. This work

demonstrated the significant hormetic effects induced by Cd in *P. aibuhitensis*. Therefore, the hormetic effects should be considered in the ecological risk assessment for the environmental pollutants.

Acknowledgments

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