



Dose-dependent responses of metabolism and tissue injuries in clam *Ruditapes philippinarum* after subchronic exposure to cadmium

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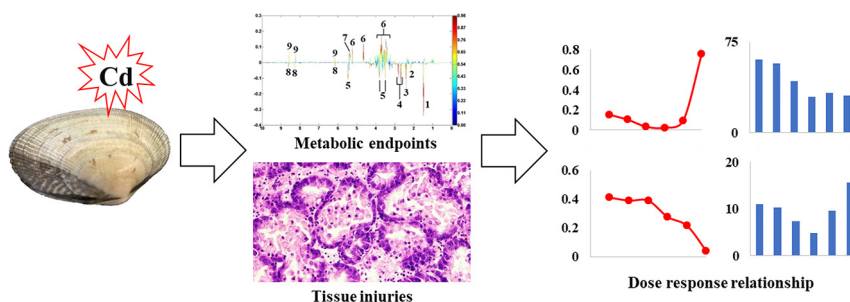
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HIGHLIGHTS

- Cd exposure resulted in both monotonic and non-monotonic responses in clams.
- The key enzymes hexokinase and citrate synthase presented monotonic decreases.
- Succinate and SDH presented typical hormetic dose-response curves.
- Hepatopancreas was more sensitive than gills to Cd in terms of injury occurrence.
- Glutamine and citrate synthase presenting monotonic curves were preferable biomarkers.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine cadmium (Cd) pollution has been globally occurring, which creates a pressing need to characterize toxicological effects and develop biomarkers for Cd. However, the dose-response relationships challenge toxicity characterization and biomarkers selection. Metabolic processes have been frequently targeted by Cd. In this work, we investigated the dose-dependent effects of Cd on metabolic endpoints in whole soft tissues as well as gill and hepatopancreas injuries in clam *Ruditapes philippinarum*, aiming to better understand the metabolic responses and develop biomarkers. Nuclear magnetic resonance (NMR)-based metabolomic analysis was conducted on clam whole soft tissues to identify metabolites. The enzymes and metabolites associated with tricarboxylic acid (TCA) cycle, glycolysis, and oxidative phosphorylation showed both monotonic and non-monotonic curves with the increase of Cd dose. In details, glutamine, glucose-1-phosphate, hexokinase (HK), and citrate synthase (CS) presented monotonic decreases with the increase of Cd dose, among which glutamine and CS were preferable biomarkers to Cd exposure based on lower benchmark dose (BMD) values. The monotonic decreases of HK and CS activities suggested Cd exposure potentially disrupted glycolysis and TCA cycle via inhibiting rate-limiting enzymes. In contrast, the non-monotonic responses of succinate dehydrogenase (SDH), alanine aminotransferase (ALT), and their substrates (succinate and alanine) were approximate to U- or J-shaped curves, suggesting the adaptive strategy of metabolic responses to different degrees of Cd stress, like induction of anaerobiosis as energy compensation. Especially, the alterations of succinate and SDH presented

Abbreviations: AIC, Akaike information criterion; ALT, alanine aminotransferase; AMP, adenosine monophosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; BCA, bicinchoninic acid; BCAA, branched chain amino acid; BMD, benchmark dose; BMDL, benchmark dose lower confidence limit; BMDs, Benchmark Dose Modeling Software; BMR, benchmark response; CS, citrate synthase; HCl, histopathological condition index; HE, hematoxylin-eosine; HK, hexokinase; G1P, glucose-1-phosphate; GGR, gamma-glutamyl transpeptidase; ICP-MS, inductively coupled plasma mass spectrometry; NMR, nuclear magnetic resonance; O-PLS-DA, orthogonal projection to latent structure with discriminant analysis; OXPHOS, oxidative phosphorylation; PCA, principal components analysis; PK, pyruvate kinase; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

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typical hormetic dose-response curves. What is more, clam hepatopancreas was more sensitive to Cd than gill in terms of injury occurrence. Overall, characterization of dose-dependent effect of Cd on metabolism and tissue injuries provides a new insight into understanding the metabolic adaptation in marine clams and risk assessment of Cd pollution.

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1. Introduction

Cadmium (Cd) pollution is regarded as one of the emerging priority issues of United Nations Environment Programme (UN Environment Programme, 2020). As a non-essential metal element for organism, Cd accumulation to a certain extent tends to induce toxicities and pose risk on health (Du et al., 2021). Marine Cd pollution has been globally occurring because of industrial effluents and river discharge. Our recent investigations on marine organisms sampled from the Bohai Sea revealed that Cd was one of the most frequently detectable metal contaminants (Ji et al., 2016; Xu et al., 2016). The increasing detection of Cd is of considerable environmental concern, which creates a pressing need to characterize toxicological effects and develop biomarkers of Cd. Though biomarkers have the potentials in monitoring and risk assessment, they also face challenges because of dose-response relationships between Cd doses and biological responses.

Dose-response is the backbone of toxicology, and the dose-response relationship is the key in understanding how many adverse effects can be induced to organisms, as well as the tolerance capacity of organisms to stressors (Agathokleous and Calabrese, 2019). In general, the relationship presents not only monotonic responses like linear, power, exponential curves, but also non-monotonic responses like U-shaped and J-shaped curves (or inverted U-shaped and inverted J-shaped curves, depending on the endpoints) (Agathokleous, 2018). Undoubtedly, the endpoints with monotonic responses are potential biomarkers to indicate stresses. However, the effects of high doses cannot be predicted by the effects observed at low doses in non-monotonic curves. What is more, as a special dose response, hormesis is a biphasic dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition (Calabrese, 2008; Agathokleous, 2018). Increasing evidence shows that hormesis is a highly generalized dose-response phenomenon in animals, plants and microbes (Calabrese et al., 2019). Ignoring dose-response relationships therefore is not the ideal methodology for not only characterizing toxicity but also selecting biomarkers.

Metabolism homeostasis plays essential roles in organism's survival and function, as well as in stress adaptation and tolerance (Sokolova et al., 2012). Metabolic processes, such as tricarboxylic acid (TCA) cycle, glycolysis, and oxidative phosphorylation (OXPHOS) are frequently identified as the targets of Cd (Sokolova et al., 2005b; Ji et al., 2019). However, these researches failed to reveal the dose-dependent effects of Cd because of lacking adequate number of Cd doses. So far, studies on dose-responses between Cd and metabolism are relatively limited. Ivanina et al. (2008) observed U-shaped dose-response curves in mitochondrial enzymes in Cd-treated oysters. Metabolomics can identify and quantify metabolic products of a biological system, and has been increasingly used in ecotoxicological studies to evaluate the toxicological effects of contaminants (Xie et al., 2020). In our recent work, metabolome profiles in *Perinereis aibuhitensis* were used to investigate the dose response of metabolites, demonstrating that Cd induced multiple dose-responses at metabolite level (Liu et al., 2019). These researches confirmed the existence of dose-dependent effects of Cd on metabolism.

Marine clam *Ruditapes philippinarum* is an economically and scientifically important bivalve. Due to its wide distribution, ease of collection, low mobility, and filter-feeding, *R. philippinarum* has been frequently used to characterize the adverse effects of multiple contaminants (Chen et al., 2018; Wu et al., 2013) and biomonitor marine pollution (Krishnakumar et al., 2018). As clams are unable to escape from stresses

and must adapt to variable environments, metabolic adaptation in clams is therefore of great importance. Dose-responses between Cd exposure and metabolic endpoints are essential for understanding how clams respond and when metabolic transitions occur. In this work, as described in the flowchart of experimental design (Fig. S1), we characterized metabolomic profiles and measured metabolic enzymes in whole soft tissues of clams from a serial of Cd exposure groups, aiming to reveal the dose-dependent effect of Cd on metabolism in clams. The calculation of histopathological condition indices (HCIs) was also conducted on clam gills and hepatopancreas to quantify the tissue injuries. Benchmark dose (BMD) is U.S. Environment Protection Agency (U.S. EPA) preferred methodology for dose-response analysis. The BMD value is determined as a dose level associated with a specified change in benchmark response (BMR) (Hardy et al., 2017). By model fitting, the BMD values of endpoints were calculated. Sensitive biomarkers were selected based on lower BMD values. We hypothesized that Cd treatments could induce not only monotonic responses but also non-monotonic responses in metabolic processes in clams. As hormesis is regarded as an overcompensation response following an initial disruption in homeostasis (Calabrese, 2008), we also hypothesized that hormesis was present during metabolism homeostasis in response to Cd stress. The investigation on dose dependence of Cd will provide a new insight into understanding the metabolic adaptation in marine clams and risk assessment of Cd pollution.

2. Materials and methods

2.1. Animals and exposure conditions

Adult clams *R. philippinarum* (shell length: 3.5–3.8 cm, White pedigree) were purchased from an aquaculture farm (Qingdao, China). The exposure experiment was conducted according to Ji et al. (2019). After transported to laboratory, clams were acclimated in aerated seawater for 5 days, and then randomly divided into 6 groups, including control group and 5 Cd-treated groups. Each group contained two replicate tanks, each of which consisted of 15 individuals in 15 L filtered seawater (FSW). Control clams were kept in FSW. Cd exposure concentrations included 3, 9, 27, 81, and 243 µg/L, of which 3, 9 and 27 µg/L were environmentally relevant (Fu et al., 2014; Zhang et al., 2010) and 243 µg/L referred to nearly 20% of 96-h LC₅₀ of Cd to *R. philippinarum* (1360 µg/L). The stock solutions of CdCl₂ were prepared by dissolving analytical reagent grade CdCl₂ in deionized water. During the acclimation and exposure periods, clams were kept in aerated seawater (25 ± 2 °C, 31 psu) under a photoperiod of 12 h light and 12 h dark, fed with *Chlorella vulgaris* of 2% tissue dry weight once daily. Exposure seawater was completely replaced by fresh seawater daily. After exposure for 14 days, 4 clams were selected for histopathological analysis on gills and hepatopancreas, and another 9 clams were selected for measurements of Cd accumulation, enzymes activities and metabolomic profiles. Of the 9 clams, the whole soft tissue of each individual was ground to powder in liquid nitrogen. Each sample was then divided into 3 parts for simultaneous measurement of Cd accumulation, enzymes activities and metabolomic profiles.

2.2. Measurement of Cd accumulation in whole soft tissues of clams

Cd accumulation in clams was measured according to the description by Wu and Wang (2010). In details, the whole soft tissue samples

($n = 9$) of clams were dried at 80 °C to constant weights, which were recorded for calculation of Cd accumulation. The dried samples were then digested in concentrated nitric acid (70%, Fisher Scientific) heated in water bath overnight. All completely digested samples were diluted with ultrapure water for the determination of Cd using inductively coupled plasma mass spectrometry (ICP-MS) technique (Agilent 7500i, Agilent Technologies Co. Ltd., Santa Clara, CA, USA). The limit of quantification (LOQ) of Cd was 0.1 ppb, which was at least two orders of magnitude lower than those detected in control clam samples. Marine mussel tissue standard reference material (GBW08571) was employed as a certified reference material for metal analysis to ensure internal quality assurance/quality control (QA/QC) practices. The recovery of target elements, as tested by three individual spiking experiments, was restricted within 95.5%–104.3% for Cd.

2.3. Metabolomic analysis

Nuclear magnetic resonance (NMR)-based metabolomics was used to unravel the alterations of metabolites in clams treated with Cd. Polar metabolites in clam whole body ($n = 9$) were extracted in 4 mL/g of methanol, 5.25 mL/g of water and 2 mL/g of chloroform as described previously and analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously (Wu and Wang, 2010).

All one dimensional ^1H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathWorks, Natick, MA, USA) and then segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. All the NMR spectra were generalized log transformed with a transformation parameter $\lambda = 1.0 \times 10^{-8}$ and subsequently data were mean-centered before data analysis. The unsupervised multivariate data analysis methods, principal components analysis (PCA), was performed to detect the global dose-dependent response profiles related to Cd exposures. The supervised multivariate data analysis methods, orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were sequentially carried out to uncover and extract the statistically significant metabolite variations related to Cd treatments. Metabolites were identified by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada). The metabolite concentrations (mM) were determined based on the concentration (0.5 mM) of internal standard, TSP.

2.4. Metabolic enzymes in response to Cd treatments

The activities of 6 typical enzymes related to metabolism, including hexokinase (HK, EC: 2.7.1.1), pyruvate kinase (PK, EC: 2.7.1.40), citrate synthase (CS, EC: 2.3.3.1), succinate dehydrogenase (SDH, EC: 1.3.5.1), gamma-glutamyl transpeptidase (GGT, EC: 2.3.2.2) and alanine aminotransferase (ALT, EC: 2.6.1.2), were measured in the whole soft tissue of clams from control and Cd-exposed groups. The whole soft tissue of clams ($n = 9$) ground under liquid nitrogen was firstly homogenized by adding normal saline or enzyme-specific homogenization buffers provided by enzyme kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of CS (Else et al., 1988), GGT (Meister et al., 1981) and ALT (Wilkinson et al., 1972) were assayed using a UV-vis spectrophotometer (U3900H, HITACHI), and the measurement of SDH (Burke et al., 1982), HK (Scheer et al., 1978) and PK (Gutman and Bernt, 1974) activities was conducted using a microplate reader (Infinite M200, TECAN) according to the manufacturer's protocols for enzyme kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The concentrations of total protein in homogenates were determined by bicinchoninic acid (BCA) method with bovine serum albumin as standard. The unit of HK, CS and SDH was defined as the activity of an enzyme per milligram protein (U/mg protein), and the unit of PK, GGT and ALT was defined as the activity of an enzyme per gram protein (U/g protein).

2.5. Histopathological observations

After immediate dissection from the clams, gill and hepatopancreas tissues ($n = 4$) were fixed in the Bouin's fixative solution for 24 h, followed by dehydrated in a progressive series of ethanol and embedded in paraffin. Tissues from control and Cd exposure groups were processed together in batches to remove artefacts. Histological sections (6–8 μm thickness) were cut from the paraffin embedded tissues and mounted on slides which were stained with hematoxylin-eosine (HE) and observed under a light microscope (Olympus BX61, Tokyo, Japan) at $\times 200$ magnification (zoom on the camera was $\times 2.5$).

The degree of pathological lesion was evaluated through the histopathological condition index (HCI) based on the weighted indices approach (Bernet et al., 1999; Costa et al., 2013). In brief, HCI was calculated by the biological significance of each surveyed alteration (weight) and its degree of dissemination (score). The weight value ranges from 1 (minimal significance) to 3 (maximum severity), and the score value ranges from 0 (no observed alteration) to 6 (diffusion). A total of 20 candidates of histopathological alterations were selected and listed in Table S1. The HCI was calculated according to the formula:

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

where I_h is the histopathological condition indices for the individual h ; w_j is the weight of the j th histopathological alteration; a_{jh} is the score of the j th alteration for the individual h and M_j is the maximum attributable value for the j th alteration (weight \times maximum score).

2.6. BMD modeling

A BMD is a dose or concentration that produces a predetermined change in the response rate of an adverse effect. This predetermined change in response is called the benchmark response (BMR). In this work, a default BMR of 1 standard deviation (relative to control) was used for all datasets, including metabolites, enzyme activities, and HCIs. By using Benchmark Dose Modeling Software (BMDs) version 3.1.1 by U.S. EPA, automated BMD modeling analysis was performed on these endpoints to fit the best model and calculate BMD values of endpoints. A series of models, including Linear, Polynomial, Power, Hill, Exponential (M2, M3, M4, M5), were used to model the datasets. Models were initialized using BMDs 3.1.1 model defaults. The selection of models was relied on the model recommendation decision logic (Fig. S2) (US EPA, 2012; Wignall et al., 2014). In brief, the model in viable bin was taken into consideration. The only candidate model in the viable model bin was of course deemed as the best-fit model. If the viable bin had more than one model, lowest Akaike information criterion (AIC) or lowest benchmark dose lower confidence limit (BMDL) was selected. If the range of BMDL values was sufficiently close (less than 3-fold different), the AIC value was used; otherwise, the BMDL value was used. If no viable model was recommended, model in questionable bin was also considered and selected according to the selection criterion of model in viable bin. The details of models and selection criteria of best-fit models were available in Supporting information.

2.7. Statistical analysis

The endpoints, including Cd concentrations, metabolites, enzymes activities, and HCIs in clams, were expressed as means \pm standard deviation and tested for normal distribution (Ryan-Joiner's test) and homogeneity of variances (Bartlett's test). One way analysis of variance (ANOVA) followed by Dunnett's test was performed on these endpoints from control and Cd-treated groups. The P value less than 0.05 was considered statistically significant. Statistical analyses were conducted using R software (Version 3.6.3).

3. Results

3.1. Cd accumulation in clams

After exposure to Cd for 14 days, Cd contents in whole soft tissues from Cd treatments ranged from 4.76 to 46.06 $\mu\text{g/g}$ dry weight (dw) with the increase of Cd dose (Fig. 1). Compared with Cd contents in control group, significant accumulation ($P < 0.05$) of Cd was observed in 9, 27, 81, and 243 $\mu\text{g/L}$ Cd-treated groups. Cd accumulation in clam whole soft tissues occurred under even environmentally relevant concentrations (9 and 27 $\mu\text{g/L}$) of Cd, which potentially imposed adverse effects on clams.

3.2. The response of metabolic enzyme activities in clams

The activities of six typical enzymes associated with metabolism were changed in clams treated with Cd. As shown in Fig. 2, the activities of SDH and ALT in clams showed nearly U-shaped curves with bottoms at Cd dose of 27 $\mu\text{g/L}$ ($P < 0.05$). GGT activity presented a J-shaped curve with bottom at Cd dose of 9 $\mu\text{g/L}$. HK activity decreased under low concentrations of Cd treatments and remained unchanged with increasing Cd dose. CS activity linearly decreased with the increase of Cd dose except for that in 243 $\mu\text{g/L}$ Cd-treated group. PK activity presented multi-phase responses similar to U-shaped curve.

3.3. The response of metabolomic profiles in clams

A total of 33 metabolites in the whole soft tissues of clams were identified and labeled in Supporting information Fig. S3. In order to investigate the global dose-dependent responses of metabolomic profiles, PCA analysis was conducted on the NMR spectral data from control and Cd-treated groups. As shown in Fig. 3, the average scores along PC1 accounted for 49.84% variation presenting an inverted U-shaped curve, followed by PC2 (20.32% variation) and PC3 (7.78% variation) showing linearly increased and U-shaped curves, respectively, suggesting multiple dose-response relationships between metabolites in clams and Cd dose.

O-PLS-DA demonstrated the significant ($P < 0.05$) metabolic differences between clam samples from control group and 5 Cd-treated groups with reliable Q^2 values of 0.364, 0.663, 0.948, 0.929, and 0.966.

The loading plots showed responsive metabolites in two typical Cd-treated groups (27 and 243 $\mu\text{g/L}$ Cd treatments) (Fig. 4). A total of 15 metabolites were identified to be changed in response to Cd treatments (Table S2). In general, the altered metabolites varied among Cd-treated groups, and the number of altered metabolites increased with the increase of Cd exposure dose. In details, Cd treatments induced up-regulation of 10 metabolites, of which glucose was commonly altered in all Cd-treated groups, and ATP was significantly changed only in 9, 27, and 81 $\mu\text{g/L}$ Cd-treated groups. Glycogen increased at 3, 9, 27, and 81 $\mu\text{g/L}$ Cd-treated groups and decreased at the highest concentration (243 $\mu\text{g/L}$) of Cd-treated group. Moreover, a set of increased metabolites, including branched chain amino acids (BCAAs), choline, glutamate, and phenylalanine were uniquely observed in 243 $\mu\text{g/L}$ Cd-treated clams. Cd treatments also induced down-regulations of 8 metabolites, including alanine, glucose-1-phosphate (G1P), AMP, glutamine, succinate, threonine, fumarate, and glycogen. G1P was the only metabolite commonly down-regulated in all Cd-treated groups. Decrease of alanine was elicited in all Cd-treated groups. Glutamine decreased only in three high concentrations (27, 81, and 243 $\mu\text{g/L}$) of Cd-treated groups.

Quantification was conducted on 7 commonly altered metabolites to evaluate the dose-dependent effect of Cd on clam metabolites (Fig. 5, Table 1). Glucose and ATP presented responses approximate to inverted U-shaped curves with top at dose of 9 $\mu\text{g/L}$, which were consistent with the curve along PC1 axis. The responsive profiles of alanine and AMP showing U-shaped curves were similar to the curve along PC3 axis. Succinate showed a typical J-shaped curve. Glutamine gradually decreased when Cd exposure dose exceeded 9 $\mu\text{g/L}$.

3.4. Histopathological alterations in clam gills and hepatopancreas

In control group, clams presented normal gills, including intact gill filaments and regular epithelial cells, which were also observed in 3 and 9 $\mu\text{g/L}$ Cd-treated clams (Fig. 6). By comparison, 27 and 81 $\mu\text{g/L}$ Cd treatments induced vacuole and reduction of epithelial cells in gill filaments. Moreover, irregular shape of gill filaments and decrease of frontal cilia and lateral cilia were found in 81 $\mu\text{g/L}$ Cd-treated clams. In the highest concentration (243 $\mu\text{g/L}$) Cd-treated group, clams showed totally abnormal gill filaments and necrosis in gills. Cd treatment elicited similar histological changes in hepatopancreas. Normal shape of digestive tubule consisting of regular epithelial cells and intact tubular

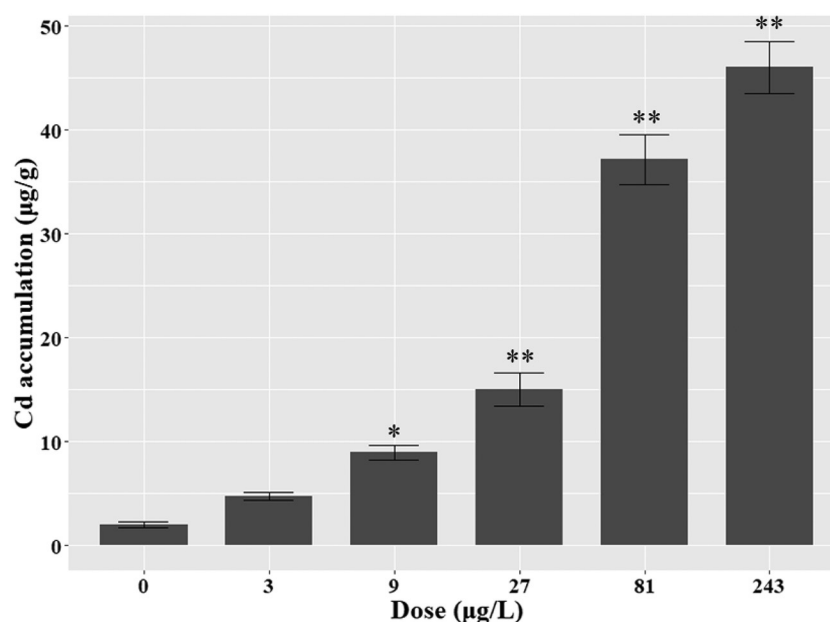


Fig. 1. The concentrations of Cd in clams. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$) between control and Cd treatment groups.

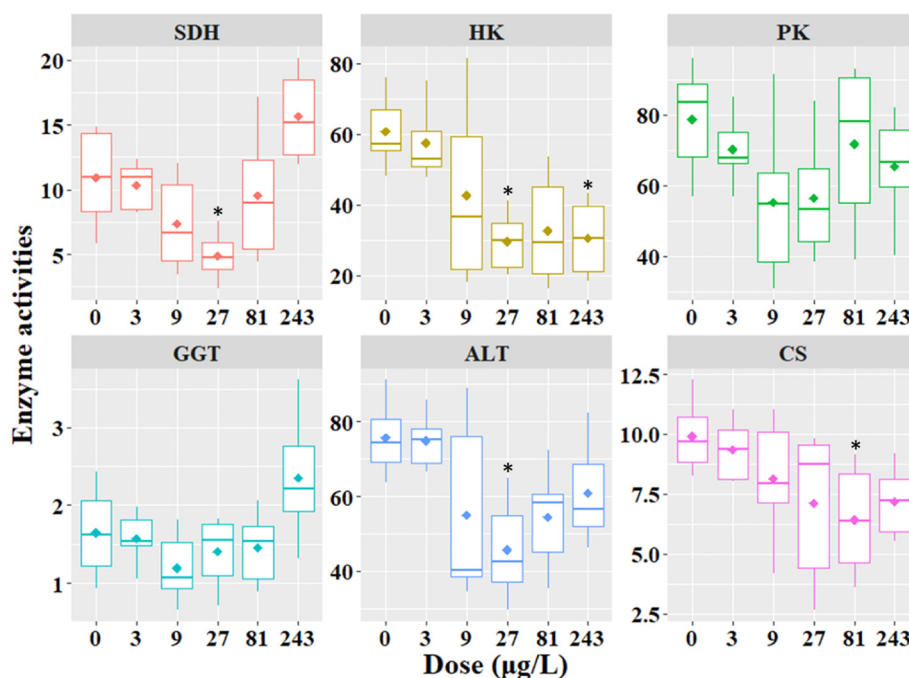


Fig. 2. The responses of metabolic enzymes in clams. The unit of HK, CS and SDH was defined as the activity of an enzyme per milligram protein (U/mg protein), and the unit of PK, GGT and GPT was defined as the activity of an enzyme per gram protein (U/g protein). Abbreviations: ALT, alanine aminotransferase; CS, citrate synthase; HK, hexokinase; GGT, gamma-glutamyl transpeptidase; PK, pyruvate kinase; SDH, succinate dehydrogenase. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$) between control and Cd treatment groups.

lumen was found in control group as well as 3 and 9 µg/L Cd-treated group. In 27 µg/L Cd-treated group, Cd treatment swelled epithelial cells and narrowed tubular lumens. Most of epithelial cells showed irregular shapes and even atrophy in clams treated with 81 µg/L Cd. In the highest concentration (243 µg/L) of Cd-treated group, necrosis of digestive tubule appeared in clam hepatopancreas, and numerous haemocytes infiltrated the damaged gland.

The pathological lesions were quantified by calculating HCLs in gills and hepatopancreas. In general, the HCLs presented an increase trend with the increase of Cd dose. Both gills and hepatopancreas did not show significant alteration of HCLs until the Cd exposure concentration reached up to 27 µg/L.

3.5. BMD estimation

In this work, BMD analysis was performed on the endpoints including metabolites, enzyme activities, and HCLs (Table 1). According to the best-fit models, BMD and BMDL values were calculated. A representative response curve of CS activities fitting to Hill model was available in Fig. S4. Of 7 metabolites, only glutamine was fitted to Hill model with BMD value of 17.87, whereas the rest failed to be fitted to viable models. Concerning the enzymes, GGT and ALT activities were commonly fitted to Exp4 model, while CS activity was fitted to Hill model. PK, SDH, and HK activities failed to be fitted to viable models under BMDs selection criteria. CS activity showed the lowest BMD value (10.67), followed by ALT activity (20.07) and GGT activity (209.92). Furthermore, the HCLs of gills and hepatopancreas were best fitted to the Exp5 and Hill models, respectively. BMD analysis demonstrated that BMD value of hepatopancreas HCLs (8.32) was less than those of gill HCLs (11.11).

4. Discussion

As it is known, exposure to pollutants may increase the energy demand for detoxification, demand repair, and/or decrease energy production (Noor et al., 2021). Severer stresses potentially induce impacts

on the aerobic pathways producing ATP by inhibiting metabolic enzymes and reducing assimilation of food. Therefore, organisms under stresses have to re-arrange metabolic strategy to meet the increasing energy demand as well as adapt the decreasing energy supply. In this work, we found that the responses of metabolites and metabolic enzymes associated with glycolysis, TCA cycle, amino acid metabolism/synthesis, and OXPHOS presented both monotonic and non-monotonic curves with the increase of Cd dose (Fig. 7). The monotonic responses suggested consistent disruption of Cd on metabolism and were potential biomarkers to Cd stress, while non-monotonic responses seemed to reflect the adaptive strategy of metabolic responses in clams.

4.1. Monotonic metabolic responses to Cd

Monotonic responses occurred in both enzymes (HK and CS) and metabolites (glutamine and G1P). HK is the initial as well as one of the rate-limiting enzymes of glycolysis (Lin and Xu, 2016). The monotonic inhibition of HK activity was explained as irreversible damage to the enzymes (Ramírez-Bajo et al., 2014). Glycolysis is highly regulated by the phosphorylation of glucose by HK, and regulation of this enzyme to a less-active form contributes to metabolic depression. CS stands as a pace-making enzyme in the first step of the TCA cycle. Diminished CS activity in Cd-treated clams was consistent with the inhibition of CS in Cd-treated oysters (Ivanina et al., 2008), suggesting that the inhibition of CS activity by Cd was probably a common strategy in bivalves to negatively affect TCA cycle. Taken together, the monotonic decreases of HK and Cd suggested that Cd might disrupt glycolysis and TCA cycle by inhibiting those pace-making enzymes.

Glutamine plays a role in a variety of biochemical functions, including protein synthesis, cellular energy, carbon donation, ammonia transport, and precursor to the neurotransmitter glutamate. Furthermore, glutamine could be converted to glutamate, which is used in the intracellular synthesis of GSH. In this work, metabolomic analysis revealed that glutamine decreased when Cd exposure concentrations exceeded 27 µg/L, suggesting the increased demand of glutamine in response to Cd exposure. Cd treatment caused depletion of glutamine was also

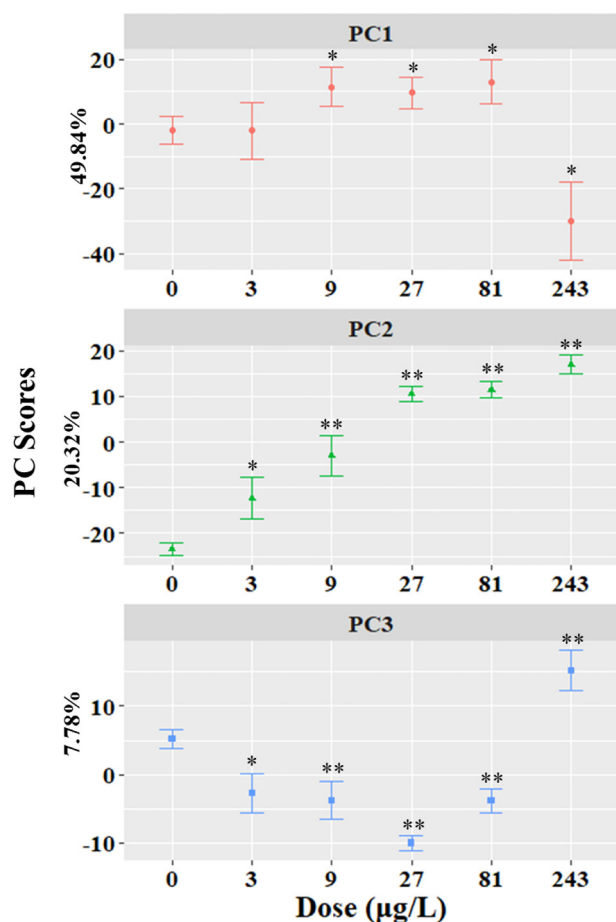


Fig. 3. The average PC scores of control and Cd-treated groups along PC1, PC2 and PC3 axes. PC scores are presented as the mean \pm standard deviation for each group. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$) between control and Cd treatment groups.

observed in rats, and supplement of glutamine enabled to protect against cadmium-induced testicular dysfunction in Wister rats (Olaniyi et al., 2020). These findings suggested that glutamine plays an essential role in Cd resistance. G1P in clams was regarded as a sensitive biomarker to Cd, since G1P decreased to a very low level when Cd exposure dose was as low as 3 µg/L. Under normal condition, energy supply is sufficiently high to cover the energy costs, and the excess of energy is deposited in storage compounds like glycogen (Sokolova et al., 2012). When organisms are suffering from stresses, energy cost increases in order to cover additional energy demands for detoxification. As glycogen degradation into glucose flows through G1P, the decreases of G1P in Cd-treated clams suggested the consistent consumption of glycogen.

4.2. Non-monotonic metabolic responses to Cd

SDH and its substrate succinate showed similar U-shaped responses with the increase of Cd dose. Linking TCA cycle and electron transport system, SDH catalyzes the oxidation of succinate to fumarate and plays a central role in mitochondrial metabolism (Jardim-Messeder et al., 2015). It has been well documented that SDH is the target of Cd (Belyaeva, 2018; Branca et al., 2020) because of its richness of reactive sulfhydryl group (Jay et al., 1991). The inhibition of SDH activity in low concentration (9 and 27 µg/L) of Cd-treated clams was in agreement with those in Cd-treated oysters (Ivanina et al., 2008). However, increases of SDH activity and succinate occurred when Cd exposure concentration ascended to some content. Succinate was one of the end products of anaerobic fermentation (Meng et al., 2016). Therefore, the

sharp increase in succinate suggested the induction of anaerobiosis in high concentration (243 µg/L) of Cd-treated group, which was consistent with the strategy of metabolic adaptation to extreme stresses (Sokolova et al., 2012). Succinate was able to protect SDH activity against cadmium ion inhibition (Jay et al., 1991), suggesting that the enhancement of SDH activity in high concentration (243 µg/L) of Cd-treated group resulted from the increase of succinate.

ALT and its substrate alanine also showed similar U-shaped curves. ALT catalyzes the transfer of amino groups from the alanine to alpha-ketoglutarate, and the conversion products are glutamate and pyruvate, of which pyruvate can be introduced to TCA cycle for energy production (Moriles and Azer, 2020). The decreases of alanine in low concentrations (9, 27 and 81 µg/L) of Cd-treated groups could be attributed to increased pyruvate demand for TCA cycle. The induction of anaerobiosis was likely responsible for the increase of alanine in high concentration (243 µg/L) of Cd-treated groups, since alanine is an end product of cytosolic anaerobiosis in mollusks (Ivanina et al., 2010). As the last step of glycolysis, PK functions as catalyzing the transfer of phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP (Gupta and Namezai, 2010). Thus the alteration of ALT and PK activities potentially influenced TCA cycle by regulation of pyruvate supply, and subsequently affected energy homeostasis in resistance to Cd stress.

GGT is encoded by *CHAC1* and involved in extracellular glutathione metabolism by transferring the glutamyl moiety to a variety of acceptor molecules, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress (Kaur et al., 2017). Located on the outer surface of plasma membrane, GGT plays key roles in GSH homeostasis by breaking down GSH and providing cysteine, the rate-limiting substrate, for intracellular de novo synthesis of GSH (Zhang et al., 2010). Our recent study has found significant up-regulation of *CHAC1* expression in high concentration of Cd treatment (data not published), which was consistent with the enzyme activity of GGT. The enhanced GGT activity in the high concentration (243 µg/L) of Cd-treated group suggested an adaptation to increased oxidative stress.

Hormesis describes a dose-response relationship separated into two dose zones, low-dose before the estimated threshold with stimulatory responses, and high-dose after the estimated threshold with inhibitory and/or adverse reaction (Agathokleous and Calabrese, 2020a). Moreover, the maximum low-dose stimulatory response is less than 2-fold of control as a rule (Calabrese et al., 2019). In this work, many endpoints such as SDH, PK, GGT, ALT, alanine, succinate, glucose, AMP, and ATP presented dose-responses approximate to U-shaped or J-shaped curves, and the maximum low-dose stimulatory responses of some endpoints were in agreement with the hormesis rule. Especially, succinate and SDH activity presented typical hormetic dose-response curves. Hormesis represents an overcompensation to a disruption in homeostasis. Low-dose effects extend the energy supply, which enables to adapt more severe stress (Agathokleous and Calabrese, 2020b). The induction of anaerobiosis indicated by a sharp increase in succinate exactly extended the energy supply and was definitely recognized as hormetic effects on metabolism. However, not all U-shaped or J-shaped curves could be regarded as hormesis despite its generalization (Agathokleous and Calabrese, 2019). Of these endpoints in this work, some U-shaped responses lacked plenty of doses with significance ($P < 0.05$) in stimulatory responses or in inhibitory responses and failed to match hormesis, which reminds us of carefully designing the exposure doses in low-dose zone.

4.3. Monotonic responses of histopathological condition indices

Stresses enable organisms to re-establish homeostasis of metabolism. However, a death program (apoptosis) or even necrosis will occur when tolerance limits are exceeded and irreversible breakdown arrives (Kultz, 2020). Chronic exposure or high doses of effectors might induce tissue injuries. In this work, injuries occurred in two

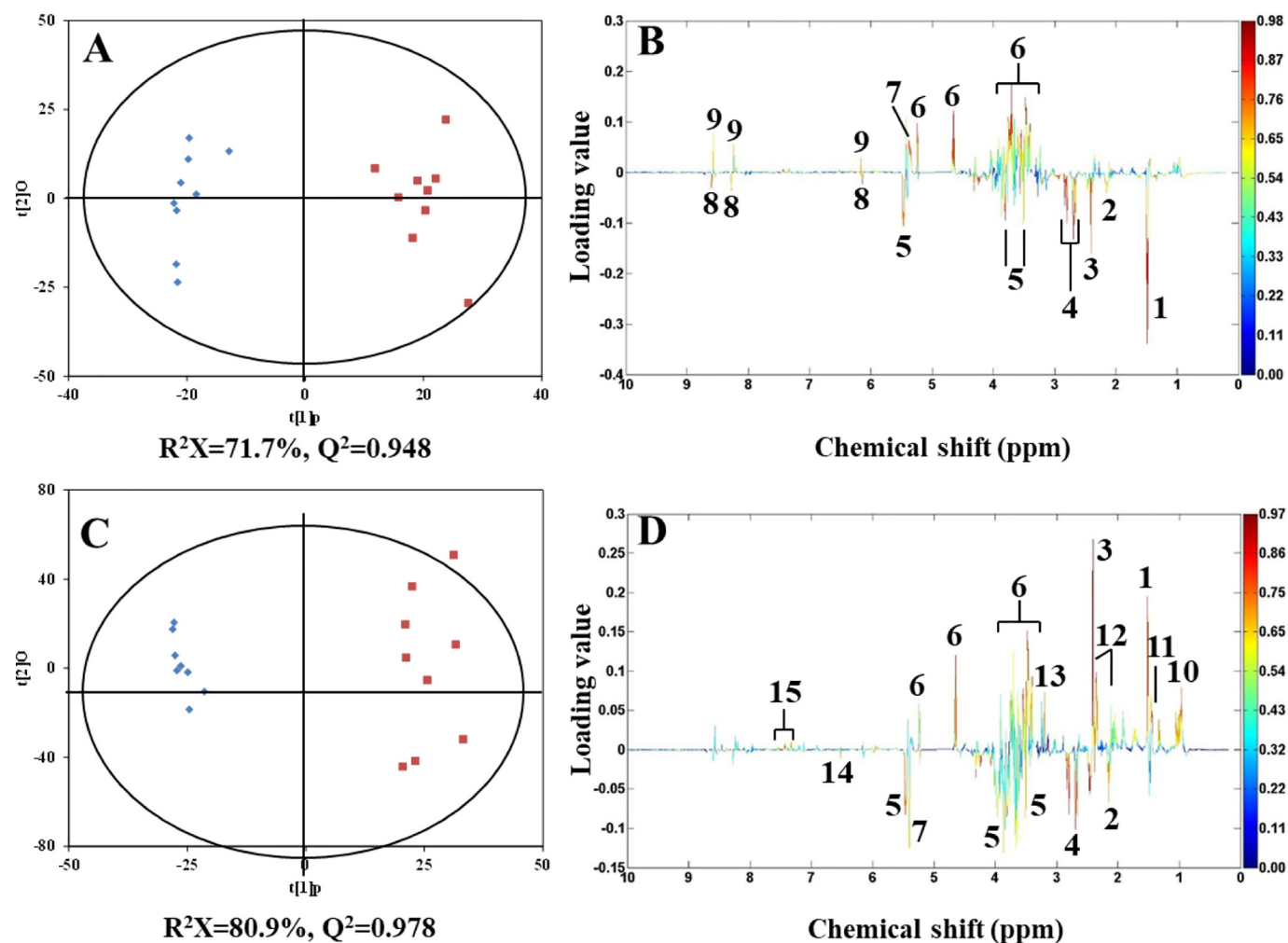


Fig. 4. OPLS-DA scores derived from ^1H NMR spectra of the whole soft body of clams from control (\blacklozenge) and Cd-treated clam groups (\blacksquare), (A) 27 $\mu\text{g/L}$ and (C) 243 $\mu\text{g/L}$ Cd treatments and its corresponding coefficient plots (B) and (D). The color map shows the significance of metabolite variations between control and Cd treatment groups. Peaks in the positive direction indicate metabolites that are more abundant in Cd treatment groups. Conversely, metabolites that are more abundant in control clams are presented as peaks in the negative direction. Keys: (1) alanine, (2) glutamine, (3) succinate, (4) unknown1 (2.68, 2.82 ppm), (5) glucose-1-phosphate, (6) glucose, (7) glycogen, (8) AMP, (9) ATP, (10) branched chain amino acids (BCAAs), (11) threonine, (12) glutamate, (13) choline, (14) fumarate, (15) phenylalanine.

typical and key organs, gill and hepatopancreas, after Cd exposure for 14 days. In bivalve, gill is an important organ for breath and filtration, and hepatopancreas plays essential roles in digestion and detoxification. Both gill and hepatopancreas are typical targets of marine contaminants (Dallares et al., 2018; Luna-Acosta et al., 2015). Cd treatments induced significant histological alterations of gill and hepatopancreas in clams treated with higher concentrations (27, 81, and 243 $\mu\text{g/L}$) of Cd. HCl evaluation enables to quantify the histopathological observations and is well applied in injury description (Costa et al., 2013). The BMD analysis on HCLs showed that the BMD value of hepatopancreas HCL was smaller than those of gill HCL, suggesting that hepatopancreas was more sensitive to Cd exposure than gill. The susceptibility of hepatopancreas to Cd could be interpreted by its involvement in metals accumulation and detoxification (Sokolova et al., 2005a; Truchet et al., 2020). Overall, hepatopancreas of clam is a preferable organ biomarker to Cd pollution.

4.4. Biomarkers selection and risk assessment

In the past decades, BMD method has been increasingly used to assess health risk of environmental contaminants. Characterization of dose-response relationships and calculation of BMD values allow for selection of preferable biomarkers. Theoretically, those endpoints

presenting monotonic curves and relatively low BMD values were potentially preferable biomarkers in clams to Cd. Accordingly, the metabolite glutamine and the enzyme CS in this work were preferable biomarkers to Cd exposure.

However, due to assumption of a monotonic dose-response relationship, BMD method has a poor capacity of model fitting on those endpoints presenting non-monotonic curves (Smetanova et al., 2015). In this work, many endpoints such as G1P, glucose, ATP, SDH, and PK failed to be fitted to a BMD dose-response curve because of nonsignificant trend or non-monotonic response to Cd exposure. Considering exposure to pollutants below the threshold where hormetic stimulation prevails, ignoring hormesis indeed causes a gap between the guideline of ecological risk assessment and reality in ecological risk assessment (Zhang and Lin, 2020). The good news is that major regulatory agencies like U.S. EPA have proposed to consider non-linear biphasic dose-response models (Agathokleous et al., 2019). The overall consideration on multiple dose responses including hormesis will improve the quality of hazard and risk assessment (Agathokleous and Calabrese, 2020a,b).

5. Conclusion

In this work, the dose-dependent effects of Cd on metabolism in clams were studied by metabolomic profiling, enzyme measurement,

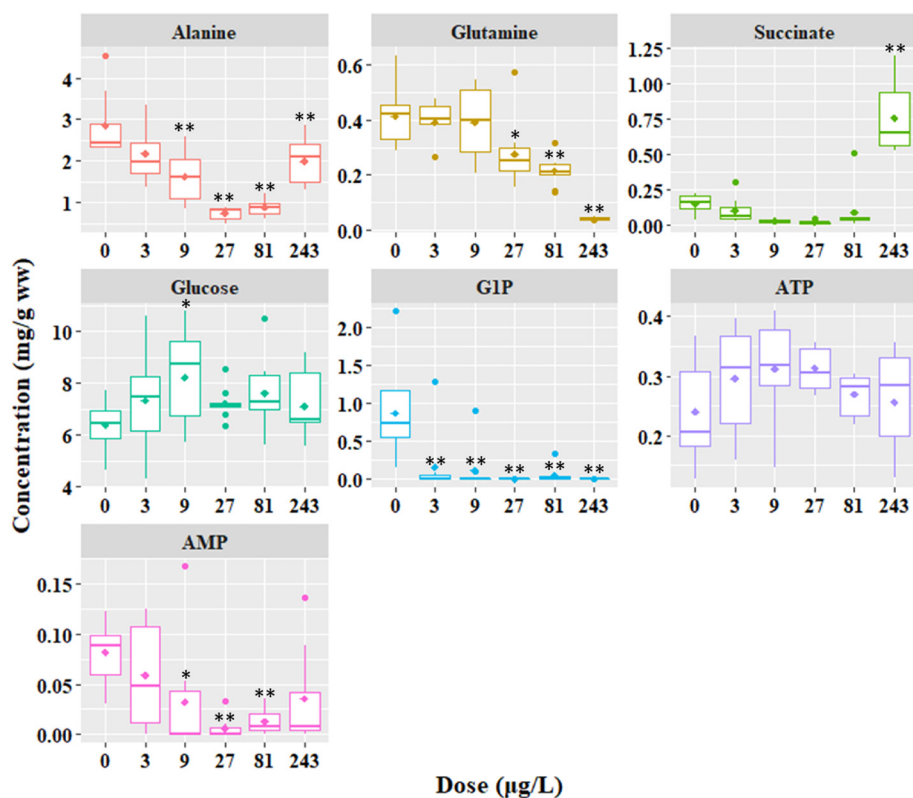


Fig. 5. The responses of metabolites in clams. Abbreviations: G1P, glucose-1-phosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$) between control and Cd treatment groups.

and quantification of tissue injury. We found that the responses of metabolites and metabolic enzymes associated with glycolysis, TCA cycle, amino acid metabolism/synthesis, and OXPHOS presented both monotonic and non-monotonic curves with the increase of Cd dose. Monotonic decreases of HK and CS activities suggested Cd exposure potentially disrupted glycolysis and TCA cycle via inhibiting rate-limiting enzymes. SDH, ALT, and their substrates presented non-monotonic responses approximate to U- or J-shaped curves, suggesting the adaptive strategy of metabolic responses to different degrees of Cd stress, like induction of anaerobiosis as energy compensation. Especially, the alterations of succinate and SDH presented typical hormetic dose-response curves. BMD analysis revealed that clam hepatopancreas tissue was more sensitive to Cd than gill tissue in terms of injury occurrence.

What is more, glutamine and CS were preferable biomarkers to Cd exposure due to their monotonic responses and relatively lower BMD values. Overall, characterization of dose-dependent effect of Cd on metabolism and tissue injuries provides a new insight into understanding the metabolic adaptation in marine clams and risk assessment of Cd pollution.

Ethical statement

All procedures were strictly performed according to the Guidelines of the Chinese Council on Laboratory Animal Care (2001), which was approved by the Animal Research Ethics Board of Chinese Academy of Sciences.

Table 1

Metabolites and enzymes ($n = 9$) in clam whole soft tissues and histopathological condition indices (HCIs) ($n = 4$) of clam gills and hepatopancreas.

Cd exposure (µg/L)	Control	3	9	27	81	243	BMD	BMDL
Metabolites (mg/g ww)								
Ala	1.52 ± 0.41	1.17 ± 0.38	0.87 ± 0.33**	0.40 ± 0.08**	0.48 ± 0.11**	1.07 ± 0.31**	PMF	PMF
Gln	0.36 ± 0.09	0.34 ± 0.07	0.34 ± 0.11	0.24 ± 0.11*	0.19 ± 0.05**	0.03 ± 0.01**	17.87	8.26
Suc	0.11 ± 0.05	0.07 ± 0.06	0.02 ± 0.01	0.02 ± 0.01	0.07 ± 0.11	0.53 ± 0.18**	PMF	PMF
Glu	6.90 ± 1.06	7.92 ± 1.92	8.88 ± 1.88*	7.82 ± 0.66	8.21 ± 1.48	7.66 ± 1.42	PMF	PMF
G1P	1.38 ± 0.98	0.26 ± 0.67**	0.18 ± 0.47**	ND**	0.09 ± 0.17**	ND**	PMF	PMF
ATP	0.73 ± 0.26	0.90 ± 0.26	0.95 ± 0.25*	0.95 ± 0.11	0.82 ± 0.11	0.78 ± 0.25	PMF	PMF
AMP	0.17 ± 0.06	0.12 ± 0.10	0.07 ± 0.11*	0.01 ± 0.02**	0.03 ± 0.03**	0.07 ± 0.10	PMF	PMF
Enzymes								
CS	9.91 ± 1.50	9.34 ± 1.30	8.14 ± 2.53	7.12 ± 3.33	6.42 ± 2.30*	7.17 ± 1.47	10.67	2.22
SDH	10.92 ± 3.82	10.32 ± 1.85	7.35 ± 3.61	4.87 ± 1.86*	9.53 ± 4.98	15.64 ± 3.53	PMF	PMF
HK	54.52 ± 17.22	53.52 ± 4.96	42.72 ± 25.68	29.66 ± 8.36**	32.76 ± 15.69*	30.65 ± 10.83*	PMF	PMF
PK	78.75 ± 15.24	66.03 ± 7.25	55.27 ± 22.14	56.41 ± 16.99	71.71 ± 22.95	65.29 ± 22.95	PMF	PMF
GGT	1.65 ± 0.58	1.51 ± 0.33	1.19 ± 0.45	1.40 ± 0.46	1.45 ± 0.47	2.35 ± 0.81	209.92	113.01
ALT	75.65 ± 22.49	74.97 ± 7.69	54.92 ± 25.79	45.65 ± 13.59*	54.41 ± 13.68	60.83 ± 13.70	20.07	9.10
HCIs								
Hepa	0.07 ± 0.03	0.08 ± 0.04	0.11 ± 0.02	0.30 ± 0.04**	0.40 ± 0.09**	0.69 ± 0.10**	8.32	5.44
Gill	0.06 ± 0.03	0.10 ± 0.05	0.13 ± 0.03	0.18 ± 0.10*	0.38 ± 0.06**	0.64 ± 0.06**	11.11	8.02

Data were expressed as mean ± SD. * and ** indicate significant differences ($P < 0.05$ and $P < 0.01$) between control and Cd treatment groups, respectively. PMF means no BMD or BMDL value because of poor model fit. The unit of HK, CS and SDH was defined as the activity of an enzyme per milligram protein (U/mg protein), and the unit of PK, GGT and ALT was defined as the activity of an enzyme per gram protein (U/g protein). Abbreviation: Ala: alanine, ALT: alanine aminotransferase, CS: citrate synthase, Hepa: hepatopancreas, G1P: glucose-1-phosphate, GGT: gamma-glutamyl transpeptidase, Gln: glutamine, Glu: glucose, HK: hexokinase, PK: pyruvate kinase, SDH: succinate dehydrogenase, Suc: succinate.

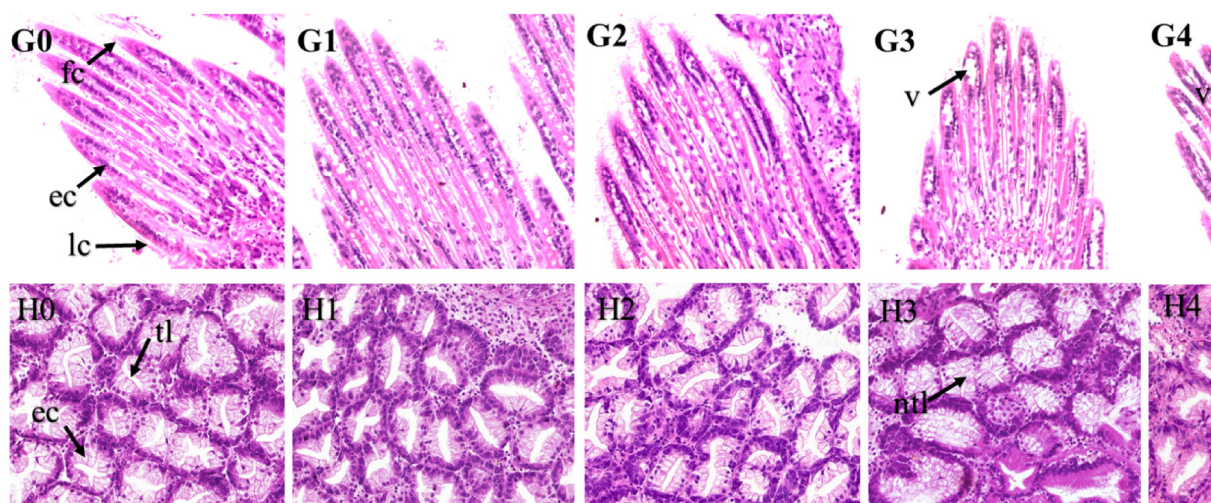


Fig. 6. Histopathological observations on clam gills and hepatopancreas. G0-G5 and H0-G5 indicate clam gills (G) and hepatopancreas (H) from control, 3, 9, 27, 81, and 243 $\mu\text{g/L}$ Cd-treated groups, respectively. Abbreviations: A, atrophy; ec, epithelial cell; fc, frontal cilia; h, haemocyte; lc, lateral cilia; n, necrosis; ntl, narrowed tubular lumen; tl, tubular lumen; v, vacuole.

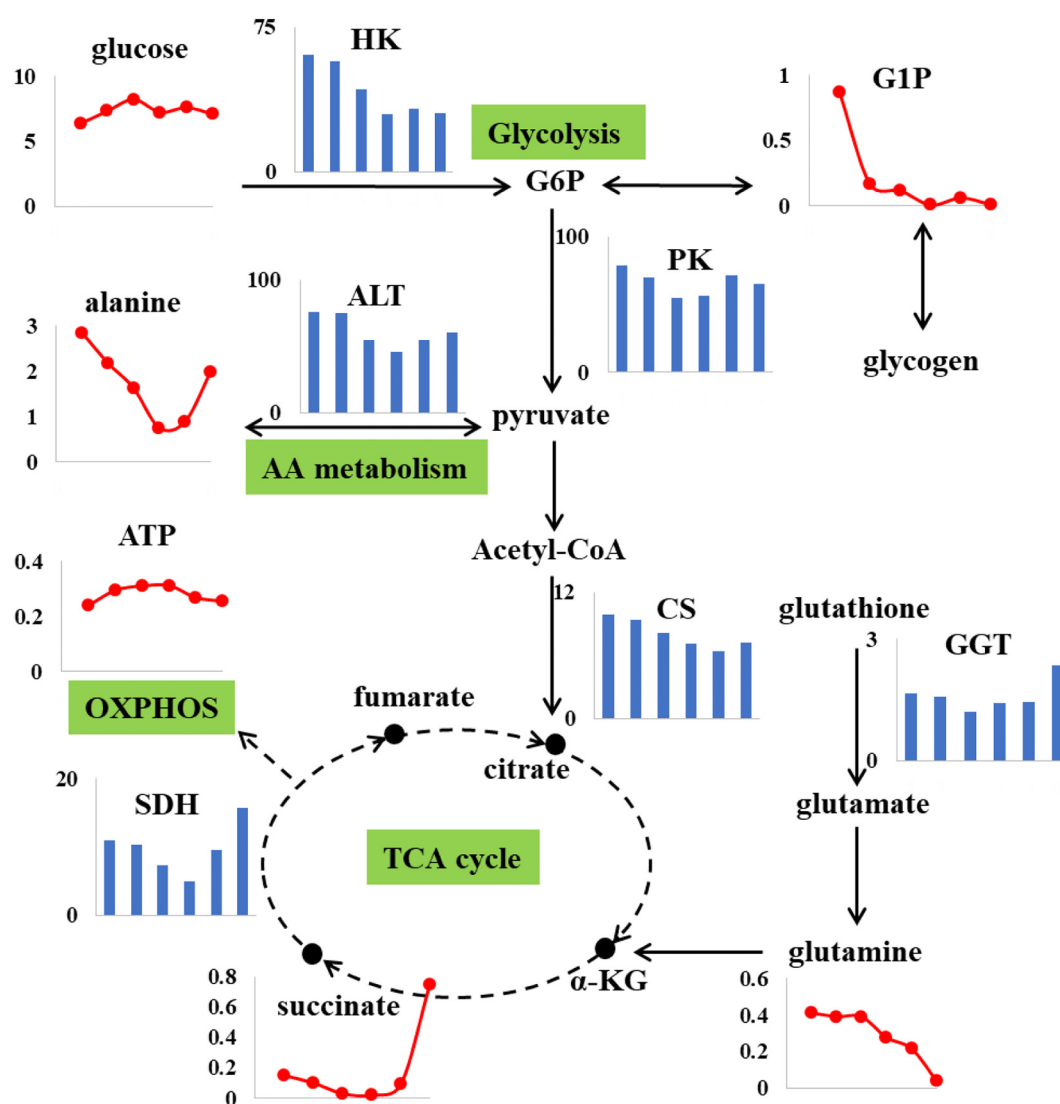


Fig. 7. The metabolic pathways related to responsive enzymes (in bar graph) and metabolites (in line graph) to Cd treatments. Abbreviations: HK: hexokinase, PK: pyruvate kinase, CS: citrate synthase, SDH: succinate dehydrogenase, GGT: gamma-glutamyl transpeptidase, ALT: alanine aminotransferase.

CRediT authorship contribution statement

Junfei Zhan: Formal analysis, Writing – original draft. **Shuang Wang:** Writing – review & editing. **Fei Li:** Writing – review & editing. **Chenglong Ji:** Funding acquisition, Writing – review & editing. **Huifeng Wu:** Funding acquisition, Writing – review & editing, Project administration.

Declaration of competing interest

We declare that we have no conflicts of interest to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.146479>.

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