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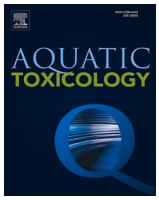

Minor metabolomic disturbances induced by glyphosate-isopropylammonium exposure at environmentally relevant concentrations in an aquatic turtle, *Pelodiscus sinensis*

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ABSTRACT

The ecotoxicological and environmental impacts of glyphosate-based herbicides have received considerable attention due to their extensive use globally. However, the potential for adverse effects in cultured non-fish vertebrate species are commonly ignored. In this study, effects on growth, indicators of functional performance, gut microbial diversity, liver antioxidant responses and metabolite profiles were evaluated in soft-shelled turtle hatchlings (*Pelodiscus sinensis*) exposed to different concentrations of glyphosate-isopropylammonium (0, 0.02, 0.2, 2 and 20 mg/L). No significant changes in growth or functional performance (food intake, swimming speed), gut microbiota, and liver antioxidant responses (SOD and CAT activities, MDA content) were observed in exposed turtles. However, hepatic metabolite profiles revealed distinct perturbations that primarily involved amino acid metabolism in turtles exposed to environmentally relevant concentrations. Overall, our results suggested that metabolite profiles may be more sensitive than phenotypic or general physiological endpoints and gut microbiota profiling, and indicate a potential mechanism of hepatotoxicity caused by glyphosate-isopropylammonium based on untargeted metabolomics analysis. Furthermore, the toxicity of glyphosate at environmentally relevant concentrations might be relatively minor in aquatic turtle species.

1. Introduction

As one of the most widely used herbicides in the world, glyphosate is largely used to control weeds in farmlands as well as in aquatic systems (Duke and Powles, 2008). Consequently, residues of glyphosate (including its metabolites) are detectable in many natural water bodies worldwide. Detected concentrations of residual glyphosate in stream surface waters have reached up to 7.6 mg/L in Europe and America (Solomon and Thompson, 2003; Contardo-Jara et al., 2009). Despite supposedly being only slightly to moderately toxic, residual glyphosate in aquatic environments has been shown to impose significant adverse effects on non-target aquatic organisms (Kier and Kirkland, 2013; Annett et al., 2014; Brovini et al., 2021). Studies have documented multiple toxicological effects (including DNA and histopathological damage, developmental malformation, growth inhibition, behavior disorder, etc.) in crustaceans, fish and amphibians exposed to glyphosate or glyphosate-based herbicide (GBH) formulations (Nwani et al., 2013; Lanctôt et al., 2014;; dos Santos et al., 2017; Sulukan et al., 2017; Hong et al., 2018). Recent evidence suggests that glyphosate should be classified as an endocrine disrupting chemical (Muñoz et al., 2021). Glyphosate exposure can disrupt hormonal receptor activity and alter hormone gene expression and synthesis in mammals (Pandey and Rudraiah, 2015; Gomez et al., 2019; Ingaramo et al., 2020), although that is rarely been explored in aquatic animals (but see de Melo et al. 2019, De Maria et al. 2022).

Phenotypic, physiological, or biochemical changes under glyphosate or GBH exposure are commonly evaluated to understand their toxicological impacts in various aquatic organisms (amphibians, Navarro-Martín et al., 2014; molluscs, Mottier et al., 2015; crustaceans, Hansen and Roslev, 2016; polychaetes, de Melo et al., 2019; fish, Ma and Li, 2015; Weeks Santos et al., 2019; Le Du-Carrée et al., 2021; turtles, Héritier et al., 2017). However, few studies have considered integrating these responses at different biological levels to evaluate overall metabolic and physiological perturbations in aquatic species under

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Table 1
Mean values (± standard errors) of actual glyphosate-isopropylammonium concentration in exposure waters.

Gly-IPA concentration treatment (mg/L)	*N*	Actual Gly-IPA concentration in exposure waters (mg/L)
0	8	< 0.001
0.02	8	0.018 ± 0.001
0.2	8	0.179 ± 0.001
2	8	1.867 ± 0.009
20	8	17.107 ± 0.212

glyphosate or GBH exposure. Omics techniques provide feasible ways to comprehensively analyze diverse physiological and metabolic upsets, and to mutually corroborate those observed responses at different levels in non-target aquatic organisms under sublethal herbicide exposure (Espín-Pérez et al., 2014; González-Ruiz et al., 2019). To date, such techniques have been applied in only a few studies (Mesnage et al., 2017), and none to our knowledge have focused on glyphosate-isopropylammonium (Gly-IPA), which represents one of many forms of glyphosate used GBHs globally.

Besides wild animals, the extensive application of herbicides likewise poses a threat to the health of cultured aquatic species, which are often raised in farms located near agricultural paddy fields. Of them, the Chinese soft-shelled turtle (*Pelodiscus sinensis*) is a commercially important aquaculture species and widely cultured in the south of China (Zhang et al., 2017). Likewise, GBHs are largely used in these areas and their residues can be frequently detected. For example, a maximum residual concentration of 15 mg/L was detected in surface waters sampled from Taihu Lake located in the Yangtze River Delta of China (Fan et al., 2011). However, very little research has considered potential ecotoxicological effects of agricultural herbicides including GBHs in cultured aquatic reptiles. Here, we integrated a series of indicators (including feeding and swimming performances, growth rate, gut microbiota, liver biochemical and metabolomic profiles) measured from *P. sinensis* hatchlings under chronic exposure to different concentrations of Gly-IPA, to assess its ecotoxicological effects in this aquatic species. Omics endpoints should be more sensitive than general phenotypic indicators and have revealed extensive physiological and metabolic changes induced by GBH exposure in some species (e.g., gut microbiota changes in bee, crab and rat, Motta et al., 2018; Yang et al., 2019; Tang et al., 2020; metabolite changes in goldfish, Li et al., 2016, 2017). Based on these findings in various organisms, diverse physiological and metabolic perturbations induced by GBH exposure may be expected in *P. sinensis* hatchlings. In this study, we were especially concerned about whether more detailed physiological and metabolic perturbations would be revealed by omics technologies.

2. Material and methods

2.1. Chemical standards

Glyphosate-isopropylammonium (Gly-IPA, CAS no. 38,641–94–0) and chemical reagents required for sample processing and liquid chromatography-mass spectrometry (LC-MS) analysis were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) or Beijing Huawei Ruike Chemical Co., Ltd (Beijing, China). Assay kits for detecting the activities of superoxide dismutase (SOD) and catalase (CAT), and the contents of total protein and malondialdehyde (MDA) in the livers of turtles were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Technical grade Gly-IPA was dissolved in distilled water to create concentrated stock solutions of 0.02, 0.2, 2, and 20 mg/mL for the exposure experiment. Stock solutions were made weekly and stored in a dark place in a refrigerator at 4 °C between water changes.

2.2. Experimental animals

We collected 140 fertilized *P. sinensis* eggs from a private farm in Huzhou city, Zhejiang province in June 2020. Eggs were transferred to the lab and incubated in plastic containers with moist vermiculite after being weighted. Containers were placed inside a climatic chamber (Binder KB240, Binder GmbH, Tuttlingen, Germany) set at 28 °C. Distilled water was added into the vermiculite as necessary to maintain a relatively constant substrate water potential. Newly hatched turtles were weighed after their yolk sac was absorbed.

2.3. Chronic exposure experiment

We selected 80 healthy hatchling turtles, and randomly allocated them to different aquaria each containing either control, 0.02, 0.2, 2 or 20 mg/L Gly-IPA (16 turtles in each treatment group, with one single turtle in each aquarium). The concentration of 20 mg/L Gly-IPA (containing 14.8 mg/L active ingredient glyphosate) is close to the maximum glyphosate residual concentration of 15 mg/L detected in some waterbodies (Fan et al., 2011). Each aquarium contained 2 L dechlorinated tap water, and concentrated stock solutions were respectively spiked into the water to achieve the different treatments before turtles were transferred. All aquaria were placed in a climate-controlled room maintained at 24 °C. During the exposure period, turtles were fed a granular commercial diet (composition: 10% water, 46% crude proteins, 3% crude lipids and 4% carbohydrates, Hangzhou Dadi Feed Co., Ltd., China) once daily. Water samples were collected from each aquarium following treatment renewals (two times a week), and analyzed for general water quality parameters and actual Gly-IPA concentrations with an ELISA Test Kit (Shanghai Hengyuan Biological Technology Co., Ltd., Shanghai, China). Water quality parameters in the experiment were as follows: temperature, 24.5 ± 0.1 (standard error, SE) °C; pH: 6.86 ± 0.02; electrical conductivity, 109.5 ± 10.1 us/cm; dissolved oxygen, 6.97 ± 0.52 mg/L; NH_3—N, 0.22 ± 0.01 mg/L. Mean values (± SE) of actual Gly-IPA concentrations were presented in Table 1. After a 30-day exposure, 40 turtles (8 replicate turtles in each treatment group) were euthanized on ice and dissected. The liver and gut of each animal was carefully removed, weighed under sterile conditions, rapidly frozen in liquid nitrogen, and stored at −80 °C before further processing (measurement of antioxidant activities, metabolites in livers, and gut microbiota). Hepatosomatic index was calculated as liver mass/body mass × 100 for each dissected individual.

The remaining 40 turtles (8 replicate turtles in each treatment group) were used to evaluate the daily food intake and growth during a 90-day exposure. Turtles were fed in the morning (08:00–09:00) as mentioned above. Two hours later, eaten pellets were counted and uneaten pellets in each aquarium were removed. Full water changes were performed twice weekly to freshen holding water, remove feces, and renew Gly-IPA treatments. Hatchling turtles were weighed every 30 days.

Swimming performance was evaluated inside a bath (120 × 10 × 20 cm^3, placed in a climate-controlled room set at 28 °C) with 10 cm depth water maintained at corresponding temperature on the day before the end of 90-day exposure. Turtles were also held in the climate-controlled room for approximately 1 h before measurement, then introduced to one end of the bath individually, and gently tapped on the mid-body with a paintbrush to encourage them to swim continuously. Synchronously, the swimming performance of turtles was recorded using a laterally positioned Panasonic HDC-SD900GK digital video camera (Panasonic Co., Japan). Each turtle was tested twice for swimming performance with a minimum of 30 min rest, and returned to their aquaria between the two trials. The video clips were later examined using the MGI VideoWave III software (MGI Software Co., Canada) for the average speed over 50 cm.

Prior to parametric statistical analyses, data were tested for normality using the Kolmogorov-Smirnov test, and for homogeneity of variances using the Levene's test. Among-group differences in individual food intake, swimming speed or hepatosomatic index of hatchling

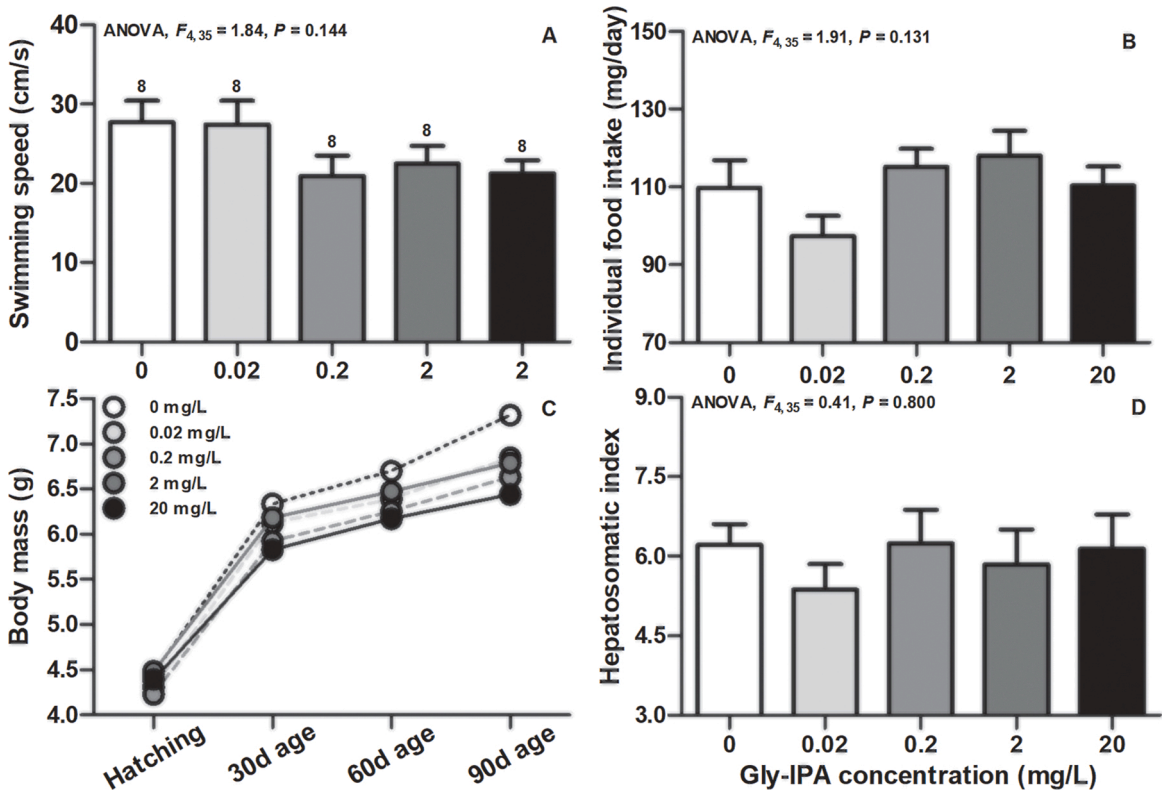

Fig. 1. Mean values (± standard errors) for swimming speed (A), daily food intake (B), post-hatching growth (C) and hepatosomatic index (D) of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings exposed to control, 0.02, 0.2, 2 and 20 mg/L glyphosate-isopropylammonium.

turtles were detected using one-way analysis of variance (ANOVA), and difference in hatchling growth performance was detected using repeated-measures ANOVA.

2.4. Gut microbiota diversity

Gut bacterial DNA was extracted using the E.Z.N.A.® stool DNA Kit (Omega Bio-tek, USA) according to the manufacturer's protocols. The quantity and quality of extracted DNA was measured using Thermo Scientific NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, USA) and 1.0% agarose gel electrophoresis, respectively. The V3–V4 variable region of the 16 S rRNA gene was amplified using universal bacterial primers, 341F (5′- CCTACGGGRSGCAGCAG −3′) and 806R (5′-GGACTACVVGGGTATCTAATC −3′). The PCRs (25 μL) contained approximately 2.5 μL DNA templates (5 ng/μL), 5.0 μL of each primer (1 μM), and 12.5 μL 2 × KAPA HiFi HotStart Ready Mix (KAPA Biosystems, USA). The PCR thermal cycling conditions were as follows: 95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s with a postamplification extension at 72 °C for 10 min. The products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA), and quantified using Thermo Scientific NanoDrop 2000 UV–Vis spectrophotometer and 2.0% agarose gel electrophoresis. Purified amplicons were sequenced on the Illumina NovaSeq PE250 platform (Illumina Inc., USA) according to the standard protocols. Gut microbiome sequencing and analysis was completed by the Hangzhou Kaitai Biotechnology Co., Ltd. (Hangzhou, China).

Raw paired end reads were assembled using PANDAseq, and obtained sequences were filtered for clean reads. After quality control, the available sequences were clustered into operational taxonomic units (OTUs) using Usearch 7.0 at the 97% similarity level. The rarefaction curves for each sample were calculated to assess the sufficiency of current sequencing depth, and OTU abundances were normalized according to the least number of reads of sample. The alpha diversity indexes including Chao and Shannon index were calculated with QIIME 1.7.0 for each sample and visualized by R software. OTUs were taxonomically classified using RDP Classifier 2.2 against the Silva (SSU v132) database with a threshold of 70% and compiled into each taxonomic level. For beta-diversity analyses, principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) based on unweighted UniFrac distance matrix were performed to test the differences in the relative abundance of OTUs among different groups. Among-group differences in alpha diversity indices and intestinal microbial composition at each taxonomic level were detected using non-parametric Kruskal-Wallis tests.

The 16S rRNA sequences in this study were deposited at the National Center for Biotechnology Information (NCBI) GenBank database under BioProject accession number: PRJNA904703.

2.5. Antioxidant response in liver extracts

A portion of liver tissue was separated, crushed with a glass rod, and then transferred into a 2 ml polypropylene centrifuge tube, in which pre-chilled 0.7% normal saline (1:10 w/v) was added. After being homogenized using an ultrasonic homogenizer (SCIENTZ-48, Ningbo Scientz Biotechnology Co., Ningbo, China), samples were centrifuged (8, 000 g) at 4 °C for 10 min, and their supernatants were transferred into a new centrifuge tube. The supernatant was diluted at 1:10 with normal saline before determining the total protein and MDA contents, SOD and CAT activities. Following the manufacturer's protocols of assay kits, total protein content was determined at 595 nm using the Bradford method, SOD activity was determined by monitoring the inhibition of the rate of cytochrome-c reduction at 450 nm using the water-soluble tetrazolium-1 method, CAT activity was determined by measuring the H_2O_2 consumption at 405 nm using the ammonium molybdate colorimetric method, and MDA content was determined at 532 nm using the thibabituric acid method (Tao et al., 2022). All assays were executed using a BioTek ELX 800 microplate spectrophotometer (BioTek Instrument Inc., USA). Among-group differences in hepatic antioxidant enzymatic

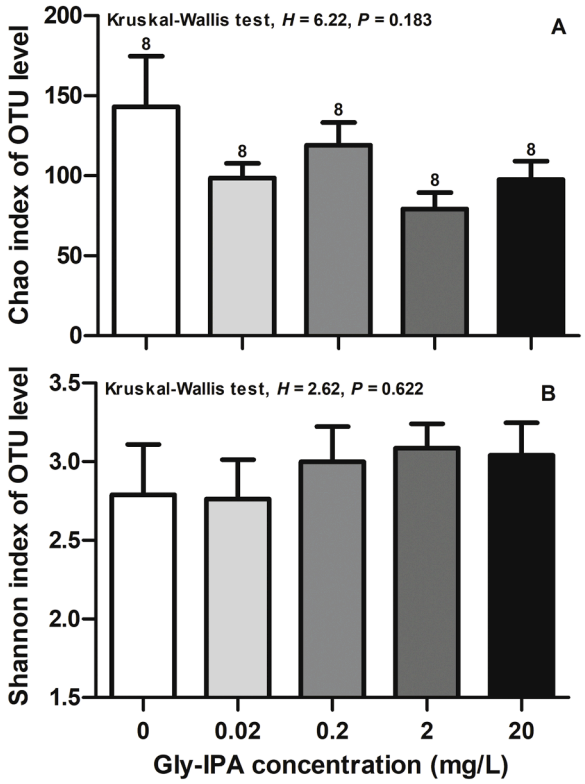

Fig. 2. Mean values (± standard errors) for the alpha diversity indexes for gut microbiota of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings exposed to control, 0.02, 0.2, 2 and 20 mg/L glyphosate-isopropylammonium.

activities and MDA content were detected using one-way ANOVA.

2.6. Metabolic profiling of liver extracts

Another portion of liver tissue (100 mg) from each sample was accurately weighed, and homogenized at low temperatures. Homogenates were vortexed and centrifuged at 4 °C for 10 min. The supernatant was transferred into a clean centrifuge tube, and dried using an Eppendorf Concentrator Plus (Eppendorf AG, Germany). The dried residue was stored at −80 °C, finally dissolved with pre-chilled 2-chlorobenzalanine/50% acetonitrile solution and filtrated through 0.22 μm filtering membrane prior to LC-MS analysis.

Chromatographic and mass spectrometry analyses were carried out on a Thermo Ultimate 3000 platform with an Acquity UPLC® HSS T3 column (Waters Acquity, USA) and Q Exactive mass spectrometer (Thermo Fisher Scientific Inc., USA), respectively. Chromatographic conditions: column temperature set at 40 °C, autosampler temperature at 8 °C, flow rate at 250 μL/min. The mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (the positive ion mode), and 5 mM ammonium formate in water and acetonitrile (the negative ion mode). After sample injection, the system was operated with the following gradient elution: 2% formic acid in acetonitrile (or acetonitrile) from 0 to 1 min, increased to 50% formic acid in acetonitrile (or acetonitrile) from 1 to 9 min, increased to 98% formic acid in acetonitrile (or acetonitrile) from 9 to 12 min, maintained at this level until 13.5 min, decreased to 2% formic acid in acetonitrile (or acetonitrile) from 13.5 to 14 min, and maintained at this level until 17 min. Mass spectrometry conditions: the spray voltage set at 3.5 kV (the positive ion mode) and −2.5 kV (the negative ion mode); sheath gas, 30 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 325 °C. The analyzer scanned over a mass range of *m/z* 81–1, 000 at 70, 000 resolution.

Raw LC-MS data were converted into mzXML format using

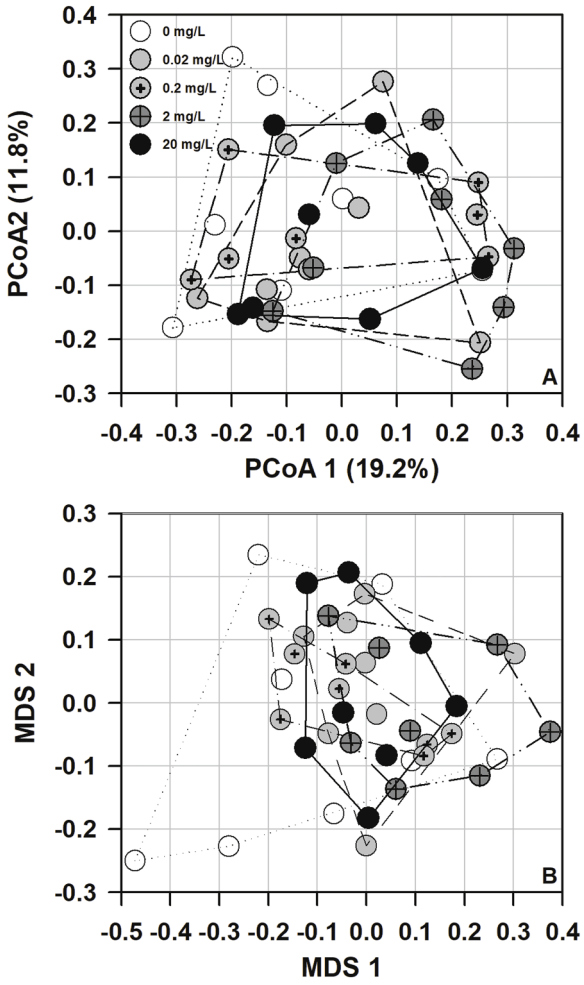

Fig. 3. Score plots for principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) for gut bacterial abundance in different groups of turtle hatchlings.

ProteoWizard v3.0.8789, processed using the XCMS software in R, and then analyzed on the MetaboAnalyst web-based platform (Pang et al., 2021). After being mean-centered and scaled to unit variance, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to compare the metabolic variations among different groups. Metabolites were identified by searching the mass spectra against available databases [i.e., Human Metabolome Database (HMDB), Metabolite Link (Metlin), MassBank Database]. Among-group differences in some key identified metabolites (using natural log-transformed spectral areas) were detected using one-way ANOVA, and post-hoc comparisons were performed with Tukey's post-hoc tests. Hepatic LC-MS analysis was completed by the Suzhou PANOMIX Biomedical Tech Co., Ltd. (Suzhou, China).

3. Results and discussion

3.1. Locomotor performance, food intake and growth rate

There were no deaths in any treatment groups during the exposure. Gly-IPA exposure was shown to have no apparent effect on locomotor (one-way ANOVA, swimming speed, $F_{4,\ 35} = 1.84$, $P = 0.144$), feeding (individual food intake, $F_{4,\ 35} = 1.91$, $P = 0.131$), or growth performances (repeated-measures ANOVA, $F_{4,\ 35} = 0.48$, $P = 0.747$) of hatchling turtles (Fig. 1). Contrarily, growth inhibition due to glyphosate or GBH exposure has been observed in other aquatic organisms, including aquatic insects, molluscs, amphibians, and fish (Mottier et al.,

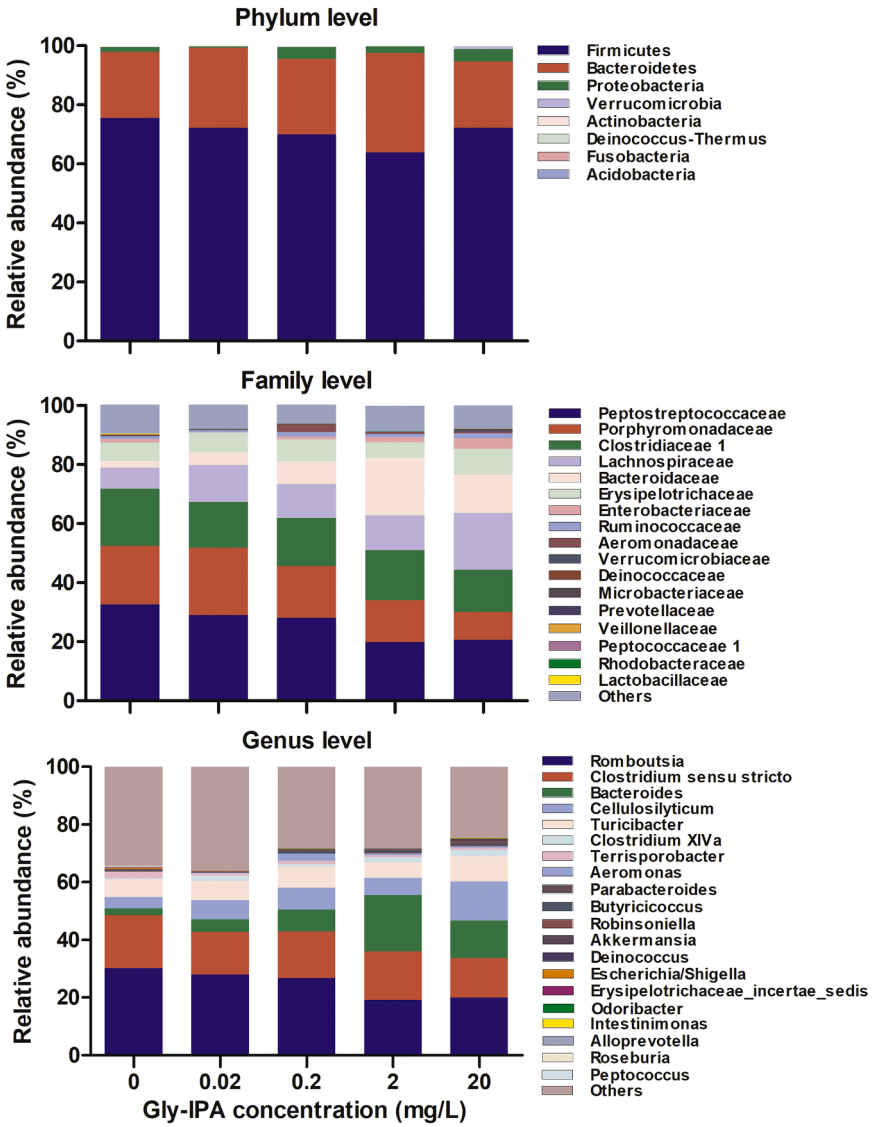

Fig. 4. Relative abundances of the gut microbiota at the phylum, family, and genus levels in different groups of turtle hatchlings.

2013; Lanctôt et al., 2014; Ferreira-Junior et al., 2017; Xu et al., 2017; Turhan et al., 2020; Lopes et al., 2022). The difference in exposure level used across studies, or sensitivity of various species to glyphosate or GBH exposure might partially explain these discrepant findings. For example, growth inhibition was observed at relatively high concentrations of glyphosate or GBH exposure in a fish *Cherax quadricarinatus* (40 mg/L, Avigliano et al., 2014), and slightly lower but still high concentrations in an insect, *Chironomus xanthus* (12 mg acid equivalent (a.e.)/L, Ferreira-Junior et al., 2017) and snail, *Pomacea canaliculate* (20 mg/L, Xu et al., 2017). Realistic concentrations of residual glyphosate measured in natural aquatic environments have not exceeded 10 mg/L, although there are a few exceptions (Fan et al., 2011). Accordingly, the impact of chronic glyphosate exposure at environmentally relevant concentrations on functional and growth performances of aquatic animals would normally be expected to be limited. In fact, chronic exposure to low glyphosate concentrations has been showed to be conducive to growth in some species (Xu et al., 2017). In this study, no obvious changes in locomotor, feeding and growth performances were observed even at 20 mg/L Gly-IPA exposure (14.8 mg glyphosate/L), potentially indicating a relatively high resistance of *P. sinensis* hatchlings to herbicide exposure compared with fish or invertebrate species.

3.2. Diversity and composition of gut microbiota

A total of 1 434 279 clean reads (approximate 35 857 reads per sample) with an average length of 410 bp were obtained from our samples. After quality control and filtration, 1 278 643 mapped reads were included in analysis (89.1%). The rarefaction curves based on the observed species and Chao index for all samples indicated that the current sequencing depth was sufficient (Fig. S1). The sequences were further clustered into 535 OTUs at the 97% similarity level. The control, 0.02, 0.2, 2, and 20 mg/L Gly-IPA exposure groups had 430, 245, 263, 178, and 228 OTUs respectively (Fig. S2). Among them, 112 OTUs were shared among these five groups, 182, 12, 24, 6 and 15 OTUs were exclusive in 0, 0.02, 0.2, 2, and 20 mg/L Gly-IPA exposure groups respectively (Fig. S2).

No significant difference in the alpha diversity of gut microbiota was observed among different treatment groups (the Chao index for diversity, Kruskal-Wallis test: $H = 6.22$, $P = 0.183$, Fig. 2A; Shannon-Wiener index, $H = 2.62$, $P = 0.622$, Fig. 2B). No significant separation of bacterial relative abundance among different treatment groups could be identified by both the PCoA and NMSD of beta diversity (Fig. 3).

At the phylum, family, and genus taxonomic levels, the bacteria with a relative abundance $> 1.5\%$ were defined as the predominant taxa for

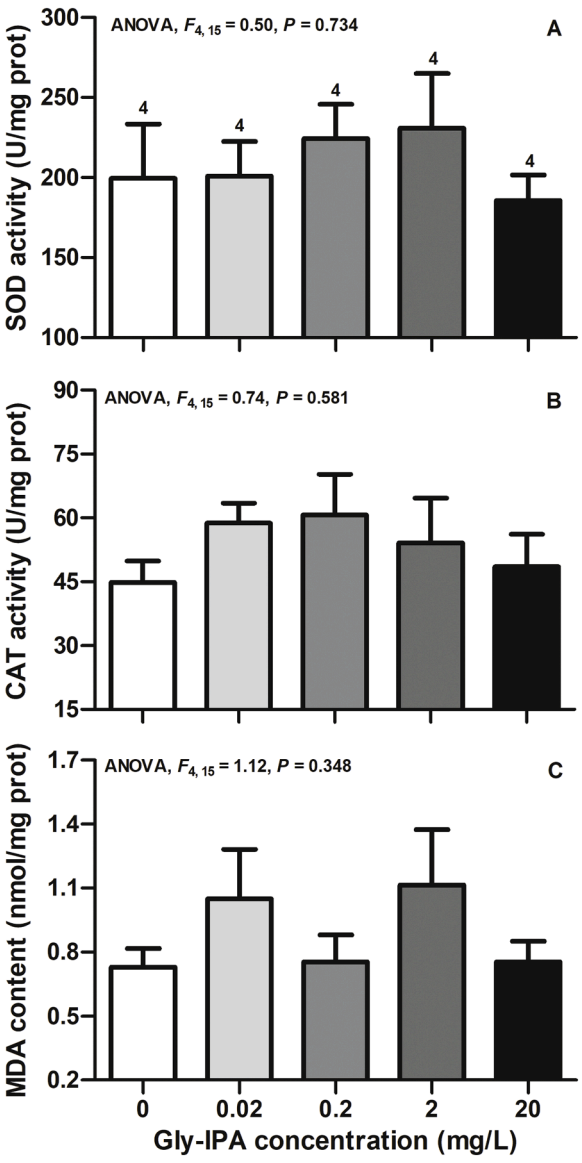

Fig. 5. Mean values (± standard errors) for SOD and CAT activities and MDA content in the livers of Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings exposed to control, 0.02, 0.2, 2 and 20 mg/L glyphosate-isopropylammonium.

ease of comparison. Further taxonomic analysis showed that Firmicutes (70.8 ± 3.5%), Bacteroidetes (26.3 ± 3.4%) and Proteobacteria (2.5 ± 0.8%) were the most predominant phyla in all samples (Fig. S3) but did not differ among different treatment groups (Kruskal-Wallis test, all $P >$ 0.322, Fig. 4). Similarly, no significant differences among groups were observed at the family level (Fig. 4). More than 20 genera were identified. Of them, *Romboutsia* (24.8 ± 2.2%), *Clostridium sensu stricto* (16.0 ± 0.8%), *Bacteroides* (9.3 ± 3.1%), *Cellulosilyticum* (7.6 ± 1.6%), *Turicibacter* (6.7 ± 0.6%), and *Clostridium XlVa* (1.5 ± 0.4%) were the most predominant genera (Fig. S3). Among-group differences in relative abundance were found in some bacterial genera, but they only accounted for a very low proportion among identified bacterial genera (e.g., *Alloprevotella*, 0.049 ± 0.047%; *Escherichia/Shigella*, 0.101 ± 0.079%, Fig. 4).

Overall, our gut microbiome data indicated a limited impact of Gly-IPA exposure on intestinal bacterial community in *P. sinensis* hatchlings. No significant change in microbial community structure was also observed in other turtle species (*Graptemys pseudogeographica*) exposed to a low concentration of GBH (10 μg a.e./L, Madison et al., 2018). Contrarily, altered gut microbiota due to glyphosate or GBH exposure has been described in other aquatic species, such as crustaceans (48.95 mg/L exposure in *Eriocheir sinensis*, Yang et al., 2019; 1 mg/L exposure in *Daphnia magna*, Suppa et al., 2020) and molluscs (100 μg/L exposure in *Mytilus galloprovincialis*, Iori et al., 2020). In general, it would be expected that herbicides like glyphosate or GBH would reduce the intestinal bacterial abundance, causing an increase in opportunistic pathogens and thereby increased mortality (Giambò et al., 2021). Furthermore, *in vitro* experiments have shown that glyphosate exposure inhibited significantly the growth of intestinal bacteria derived from a marine turtle, *Chelonia mydas* (Kittle et al., 2018). However, *in vivo*, such inhibitory effect of glyphosate exposure on intestinal bacteria were not apparent in *P. sinensis* in this study, nor in *G. pseudogeographica* previously (Madison et al., 2018).

3.3. Hepatic antioxidant enzymatic activities and MDA content

The size of dissected livers did not differ among treatment groups (hepatosomatic index, one-way ANOVA, $F_{4, 35} = 0.41$, $P = 0.800$, Fig. 1). No significant impacts of Gly-IPA exposure on hepatic enzymatic activity (SOD, $F_{4, 15} = 0.50$, $P = 0.734$; CAT, $F_{4, 15} = 0.74$, $P = 0.581$) and MDA content ($F_{4, 15} = 1.12$, $P = 0.384$) could be observed in this study (Fig. 5). As we know, SOD and CAT are important antioxidant enzymes and participate in the detoxifying process under pollutant stress, while MDA levels can indirectly reflect the status of oxidative tissue injury. Accordingly, altered SOD and CAT activities, and MDA content are often reported in various tissues from aquatic organisms exposed to environmental pollutants, including herbicides (Lushchak et al., 2009; Murussi et al., 2016; de Moura et al., 2017). Significant changes in SOD and CAT activities, or MDA content have been observed in some aquatic species exposed to glyphosate or GBH (Nwanie et al., 2013), whereas no such effects have been reported in other species (Mottier et al., 2015; De Melo Tarouco et al., 2017; Sobjak et al., 2017). In a field study, varied SOD and CAT activities were detected in a turtle species (*Mauremys leprosa*) sampled from glyphosate-contaminated natural water bodies (Héritier et al., 2017), which is inconsistent with the unremarkable alterations in our Gly-IPA exposed *P. sinensis* hatchlings. Besides antioxidant enzymes, other molecules (such as glutathione, ascorbic acid, biliverdin, etc.) are also involved in the antioxidant defense system (de Melo Tarouco et al., 2017), however possible alterations in these non-enzymatic antioxidants were not evaluated in this study. Discrepant results of antioxidant response to glyphosate exposure from different studies might be explained partially by differential antioxidant potential among tissues, species, or habitats.

3.4. Hepatic metabolite profile

Unsupervised PCA analysis of hepatic metabolite profiles showed a modest degree of separation among different treatment groups, with the first two components explaining more than 30% of the total variance of the raw data (31.0% and 46.8% in the positive and negative ion modes, Fig. 6). Supervised PLS-DA was used to further characterize these differences, and revealed a more obvious separation among different groups, with the first two latent components respectively explaining 24.8% and 40.2% of the total variance (Fig. 6). Differentially expressed metabolites induced by Gly-IPA exposure at different concentrations were identified by searching and comparing the information from available databases (such as MassBank, Metlin, and HMDB). These metabolites were primarily involved in amino acid metabolism (such as phenylalanine, histidine, valine, glutamine, glutamic acid etc.,). Some amino acids increased with increasing Gly-IPA concentration (phenylalanine and histidine), while others decreased (valine and glutamic acid), and others exhibited a non-linear dose-response (glutamine and asparagine, Fig. 7). These results revealed that, despite no observed phenotypic, oxidative stress or microbiome alterations, hepatic metabolomic profiling was sensitive to glyphosate-induced perturbations.

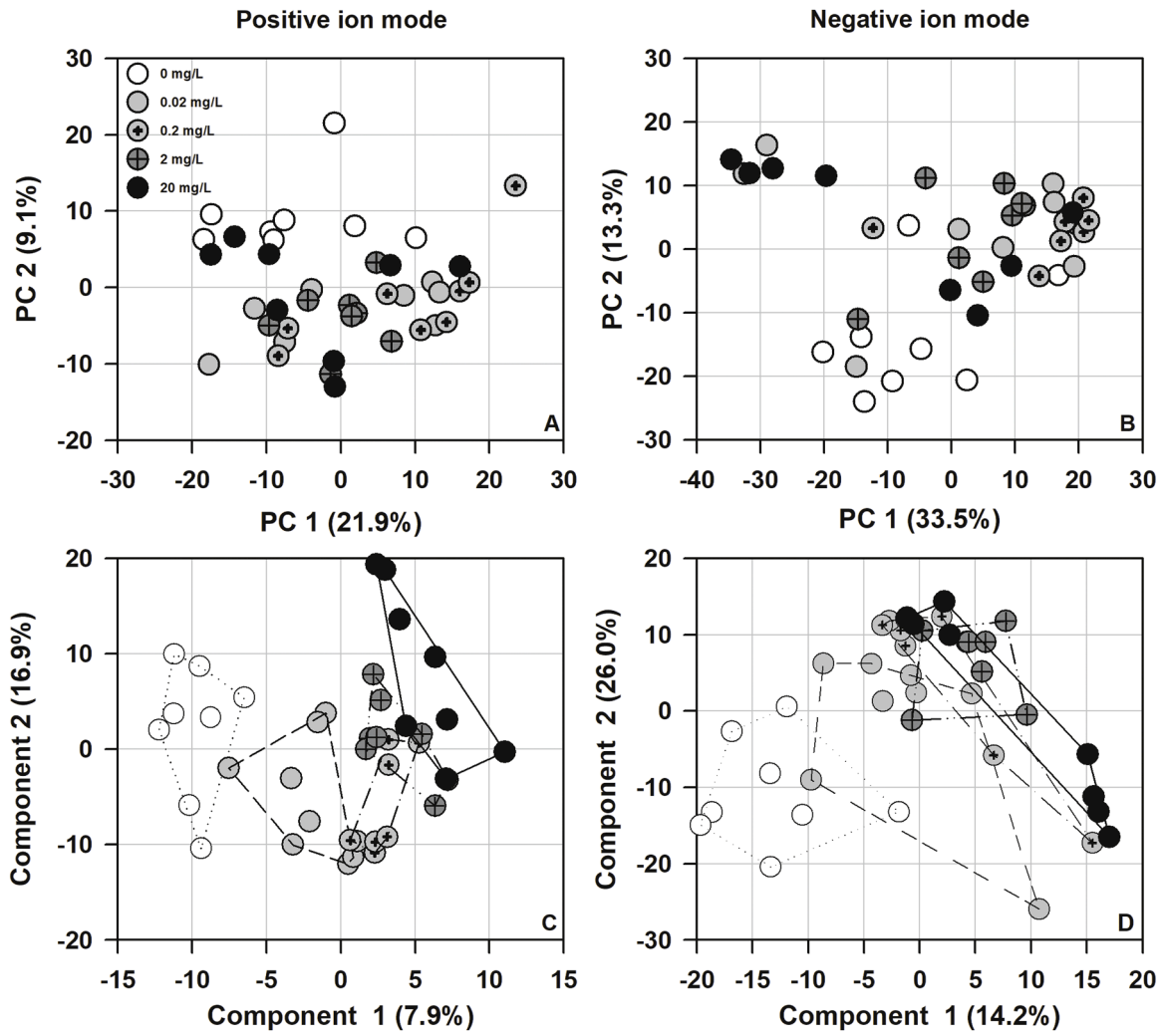

Fig. 6. Score plots for principal component analysis [PCA, in the positive (A) and negative (B) ion mode] and partial least squares discriminant analysis [PLS-DA, in the positive (C) and negative (D) ion mode] showing separation of treatment groups, respectively.

With their varied physiological roles, effects on amino acid levels have the potential to influence multiple metabolic processes. For example, elevated glutamine levels were observed in the brains of glyphosate-exposed goldfish (*Carassius auratus*) and believed to be associated with neurological effects (Li et al., 2016). Additionally, the levels of branch-chain amino acids (BCAAs) were significantly decreased in livers of glyphosate-exposed goldfish and suggested to reflect a protective response under toxicant-induced stress (Li et al., 2016). We did not measure brain metabolites, but hepatic glutamine levels were shown to be significantly reduced in Gly-IPA exposed *P. sinensis* hatchlings, which is consistent with observations in glyphosate-exposed goldfish (Li et al., 2016). Similarly, we observed decreased hepatic valine in exposed turtles, although there were no changes to other BCAAs in the present study.

Levels of several metabolites associated with energy metabolism were shown to be significantly altered in glyphosate-exposed goldfish (Li et al., 2016, 2017). We similarly observed several changes to hepatic metabolites associated with aspects of energy metabolism in this study. Creatinine showed a clear dose-response, with decreasing levels at higher Gly-IPA concentrations, potentially indicating increased renal functioning in exposed turtles (Salazar, 2014) as a mechanism to clear the herbicide from the body. The dose-dependent increase in creatinine aligns with expectations based on prior studies of glyphosate toxicity to rats (Nozdrenko et al., 2021). Contrarily, creatine levels were altered in a non-monotonic pattern in livers of exposed turtles, with a similar pattern observed for lactate. Viewed broadly, altered levels of creatine and lactate are consistent with prior reports that glyphosate exposure upsets energy reserves in muscles (Nozdrenko et al., 2021). The disparity in dose-response between creatinine and creatine may be explained by the consumption of creatine as an adaptive mechanism to decrease circulating lactate (Oliver et al., 2013). Furthermore, altered energy metabolism is also consistent with the observed increase in uridine in turtles exposed to 2 and 20 mg/L GLY-IPA, since increased uridine has been linked to ATP depletion (Zhang et al., 2020).

Increased uridine may also suggest an impact of Gly-IPA exposure on lipid metabolism (Zhang et al., 2020). While we did not actively measure a full suite of lipids in this study, the accumulation of lipids is indeed supported by increased levels of the organic fatty acid linoleic acid in livers of Gly-IPA-exposed turtles (Fig. 7). This was further consistent with evidence that fatty acid metabolism is downregulated in mice exposed to glyphosate, resulting in increased liver fat (Ford et al., 2017). We also observed reduced hepatic choline in Gly-IPA-exposed turtles and, while decreased choline did not follow a clear dose-response, deficiency of this essential nutrient is known to influence lipid metabolism and cause lipid accumulation in various tissues, including the liver (Minami et al., 2019).

4. Conclusion

In this study, a series of toxicological endpoints were used to

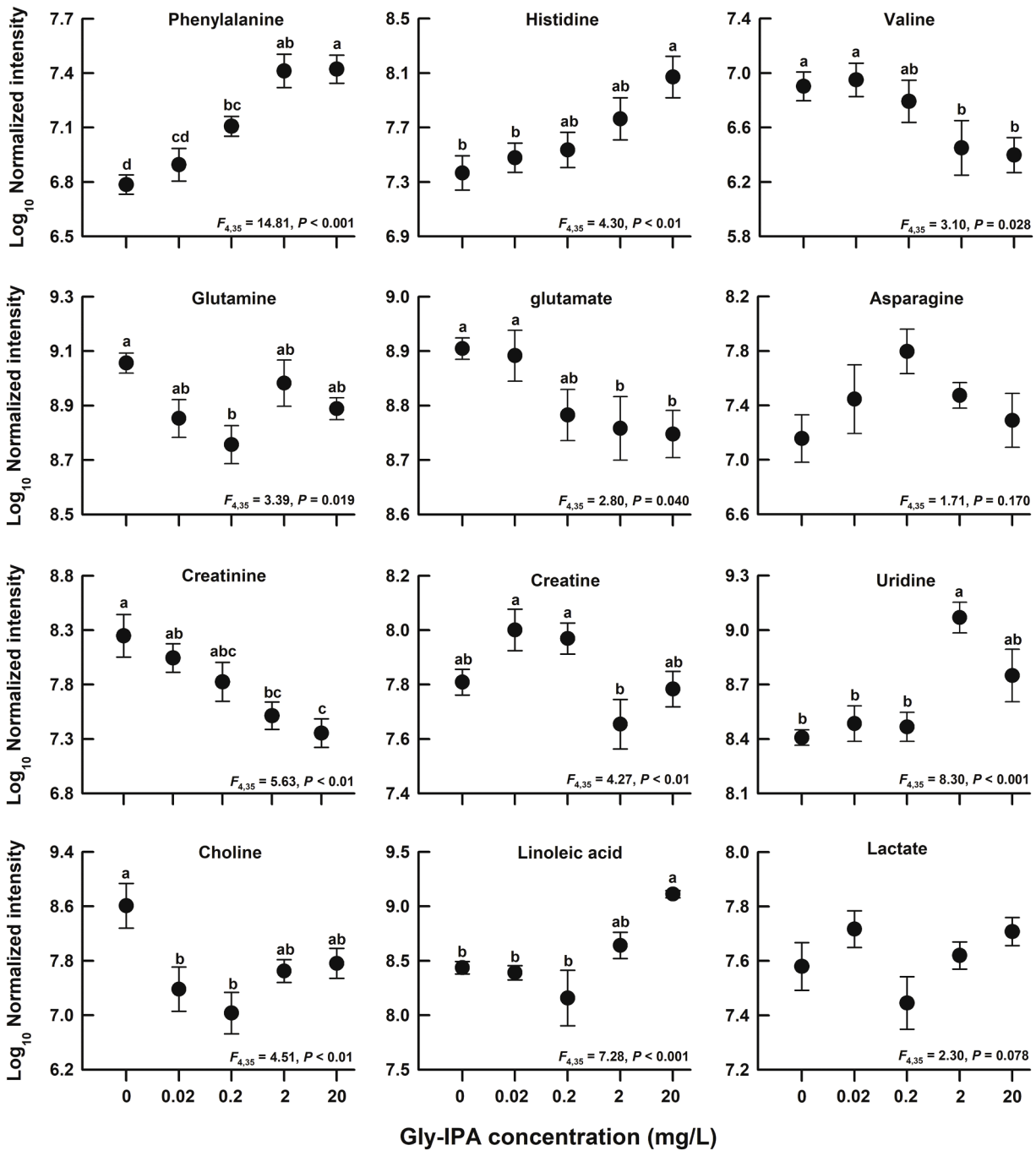

Fig. 7. Mean values (± standard errors) for some identified metabolites in the livers of Chinese soft-shelled turtle (*Pelodiscus sinensis*) hatchlings exposed to control, 0.02, 0.2, 2 and 20 mg/L glyphosate-isopropylammonium. Different letters indicate groups that differ significantly with $\alpha = 0.05$ ($a > b > c >$ d).

evaluate potential toxic effects caused by chronic GBH exposure at environmentally relevant concentrations in an aquatic turtle, *P. sinensis*. General phenotypic, physiological endpoints (e.g., locomotor ability, food intake, growth rate, antioxidant response), and gut microbiota profiles did not detect glyphosate-induced functional or physiological disorders in this species. Metabolite profile appeared to be more sensitive than these other endpoints, with untargeted metabolomics analysis revealing observable alterations in various hepatic metabolites, reflecting a potential mechanism of hepatotoxicity caused by GBH exposure. Overall, compared with other aquatic species (e.g., molluscs, crustaceans, amphibians, and fish), only slight toxic effect of GBH exposure at environmentally relevant concentrations were observed in this study, probably reflecting a relatively higher resistance to glyphosate or GBH exposure in this aquatic turtle species. However, this study was only conducted over a period of three months. Potential long-term consequences of metabolic disorders induced by glyphosate exposure in aquatic turtles still warrant investigation in future studies.

CRediT authorship contribution statement

Qin-Yuan Meng: Investigation, Software, Writing – original draft. **Chun-Quan Kang:** Methodology, Investigation, Data curation. **Wei Dang:** Data curation, Validation. **Steven D. Melvin:** Writing – review & editing. **Hong-Liang Lu:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106415.

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