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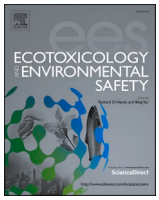

Metformin exposure altered intestinal microbiota composition and metabolites in amphibian larvae

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ABSTRACT

The antidiabetic pharmaceutical metformin (MET) is largely unmetabolized by the human body. Its residues are readily detectable in various aquatic environments and may have adverse impacts on the growth and survival of aquatic species. To date, its toxicological effects have scarcely been explored in non-fish species. Here, we exposed the tadpoles of black-spotted pond frog (*Pelophylax nigromaculatus*) to different concentrations (0, 1, 10 and 100 μg/L) of MET for 30 days and measured the body size, intestinal microbiota and metabolites to evaluate potential effects of MET exposure in amphibian larvae. MET exposure did not affect the growth and intestinal microbial diversity of tadpoles. However, intestinal microbial composition changed significantly, with some pathogenic bacteria (e.g., bacterial genera *Salmonella*, *Comamonas*, *Stenotrophomonas*, *Trichococcus*) increasing and some beneficial bacteria (e.g., *Blautia*, *Prevotella*) decreasing in MET-exposed tadpoles. The levels of some intestinal metabolites associated with growth and immune performance also changed significantly following MET exposure. Overall, our results indicated that exposure to MET, even at environmentally relevant concentrations, would cause intestinal microbiota dysbiosis and metabolite alteration, thereby influencing the health status of non-target aquatic organisms, such as amphibians.

1. Introduction

A variety of man-made pharmaceuticals are now administered to treat various animal and human diseases. Many pharmaceuticals cannot be completely metabolized by organisms, with a considerable portion of them being discharged into aquatic and soil environments (Adeleye et al., 2022; Hernández-Tenorio et al., 2022). The residues of various pharmaceuticals, as well as their derivatives, are readily detectable in different aquatic environments worldwide, and their levels are considered to be growing continuously (Elizalde-Velázquez and Gómez-Oliván, 2020; Hernández-Tenorio et al., 2022). Pharmaceutical residues in aquatic environments may cause adverse effects on non-targeted organisms, and are now considered as emerging pollutants (Shao et al., 2021; Adeleye et al., 2022). Metformin (MET) is the most widely used pharmaceutical for treating type 2 diabetes, which threatens the health of hundreds of millions of people worldwide. The major portion of MET taken by diabetic patients is not completely metabolized when passing through the body and is released into natural aquatic environments (Rena et al., 2017; Ambrosio-Albuquerque et al., 2021). In surface water, the concentration of MET varies from 12 ng/L to 33.6 μg/L (Ghoshdastidar et al., 2015; Yao et al., 2018). Yet the MET concentration in wastewater samples from hospital and urban sewage frequently exceeds 100 μg/L (Trautwein et al., 2014; Ambrosio-Albuquerque et al., 2021).

The adverse effects of pharmaceutical (including MET) residues in water bodies on various aquatic organisms have become an increasing focus of attention. Previous studies showed that MET exposure could significantly affect the survival, induce endocrine disruption and oxidative stress, cause metabolic dysregulation and behavioral disorder, disrupt embryogenesis, reduce fecundity and delay growth in fish (Niemuth and Klaper, 2015, 2018; Jacob et al., 2018; Ussery et al., 2018; Lee et al., 2019; Elizalde-Velázquez et al., 2021, 2022). Recently, the application of various omics techniques contributes to further revealing extensive physiological alterations in fish and amphibian larvae (Melvin et al., 2017; Rogall et al., 2020; Phillips et al., 2021; Blackwell et al., 2022). No obvious or only minor metabolic and gene expression changes

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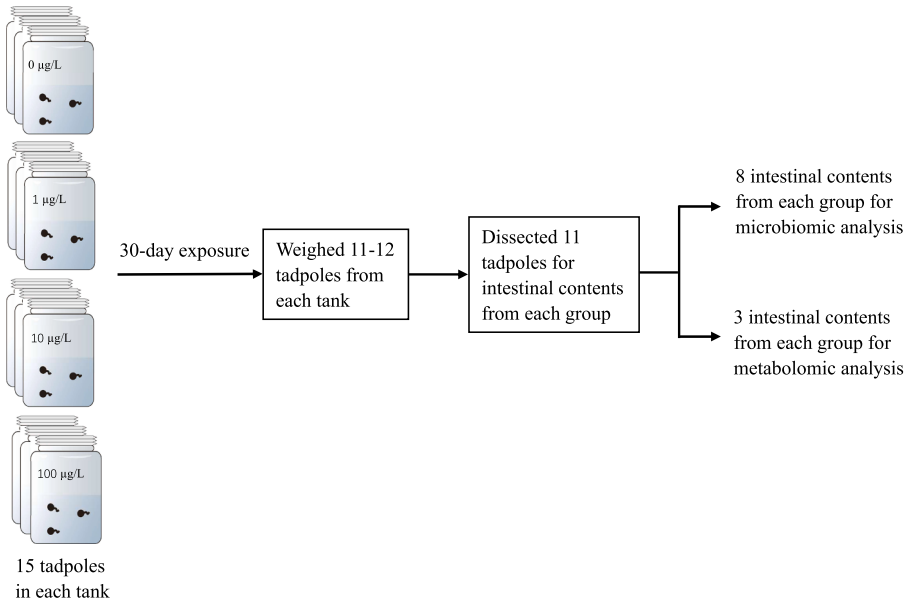

Fig. 1. The processes for the collection and treatment of tadpole intestinal samples.

were revealed by transcriptomic and metabolomic profiling after exposure to environmentally relevant concentrations of MET (Melvin et al., 2017; Phillips et al., 2021; Blackwell et al., 2022). Intestinal microbiota can play an important role in regulating host metabolism and has become a topic of great interest in aquatic toxicological studies (Evariste et al., 2019). The findings obtained from studies considering intestinal microbiota responses to MET exposure were divergent in aquatic organisms. In fish species, for example, obvious adverse impacts were found in some studies (Jacob et al., 2018; Rogall et al., 2020), but not in others (Parrott et al., 2022). More toxicological testing of MET conducted on other species is thus necessary to obtain a comprehensive understanding of its potential impact on intestinal microbiota and associated metabolites in aquatic organisms.

Amphibian populations worldwide are declining rapidly, and the impact of environmental (including pharmaceutical) pollution is considered an important factor associated with this decline (Mann et al., 2009). Toxicological studies on amphibians should be vital for understanding the underlying cause of population declines. The black-spotted pond frog, *Pelophylax nigromaculatus*, is a common amphibian species that is widely distributed in East Asia and is a suitable model animal for toxicological testing (Zhang et al., 2018; Huang et al., 2022). Here, we exposed *P. nigromaculatus* tadpoles to different concentrations of MET (1, 10 and 100 μg/ L) for 30 days and measured the changes in intestinal microbiota composition, as well as in metabolite levels, after MET exposure to assess its potential toxicological effects on amphibian larvae. Considering these findings from previous studies on other aquatic species, such as fish *Salmo trutta* and *Pimephales promelas*, exposed to MET (Jacob et al., 2018; Rogall et al., 2020; Parrott et al., 2022), the composition of intestinal microbiota and some intestinal metabolite levels would be changed in MET-exposed tadpoles.

2. Material and methods

2.1. Chemical compounds and animals

Metformin hydrochloride (> 98% purity, CAS no. 1115–70–4) and chemical reagents required for sample processing were purchased from the Aladdin or Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Metformin hydrochloride was dissolved in distilled water to prepare stock solutions.

Tadpoles of *P. nigromaculatus* at Gosner developmental stage 20 were obtained from a frog aquaculture farm located on the outskirts of Jingzhou (Hubei Province, China), transported to the laboratory in Hangzhou, and maintained in aquaria (110 × 70 × 55 cm^3) filled with dechlorinated tap water to a depth of 30 cm. The aquaria were placed in a room for the artificial simulation of atmospheric phenomena set at 25 ± 1 °C with a photoperiod of 12 h light and 12 h dark, and the tadpoles were fed with Sera mini bit food. The exposure experiment started after the majority of tadpoles had reached Gosner stage 26. A total of 180 tadpoles were randomly selected and allocated to different tanks each containing 0 (serving as the control group), 1, 10 and 100 μg/L MET (3 replicate aquaria, 45 tadpoles in each treatment group). The exposure concentrations of MET in this study were selected with reference to previous studies in fish (ranging from 1 to 100 μg/L, Crago et al., 2016; Ussery et al., 2018; Elizalde-Velázquez et al., 2021). Each tank contained 3 L of dechlorinated tap water and housed 15 tadpoles, with gentle aeration using a mini aquarium pump (Sensen Group Co., Ltd., Zhoushan, China) to maintain oxygen levels (Lu et al., 2021). An equal volume of stock solutions or water (for the control group) was spiked into tanks before tadpoles were introduced. Quality parameters of experimental water were as follows: temperature, 24.4 ± 0.1 °C; pH: 7.28 ± 0.07; electrical conductivity, 129.4 ± 4.1 μs/cm; dissolved oxygen, 6.64 ± 0.08 mg/L; NH_3-N, 0.12 ± 0.02 mg/L. Tadpoles were fed twice daily, and water was changed twice weekly. After 30-day exposure, 11–12 tadpoles were selected randomly from each tank, placed individually in an empty Petri dish and weighed to the nearest 0.0001 g on a Mettler balance (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) after the excess water on the animal's body had been gently blotted with tissue paper, to assess the variation in body size. Of them, 11 tadpoles were randomly selected from each treatment (from different tanks), euthanized on ice and dissected for intestinal contents (Fig. 1), which were then stored at − 80 °C. Tadpole dissection was carried out on an Airtech SW-CJ-1FD clean bench (Suzhou Antai Airtech Co., Ltd., Suzhou, China), which was sterilized with alcohol wipes and by irradiation with ultraviolet light for 30 min before the animals were placed on it. The animal experimental procedures were approved by the Animal Care and Ethics Committee of Hangzhou Normal University.

2.2. Intestinal microbiomic analysis

Eight samples of the intestinal contents from each treatment group were used for microbiomic analysis (Fig. 1). The detailed procedures of

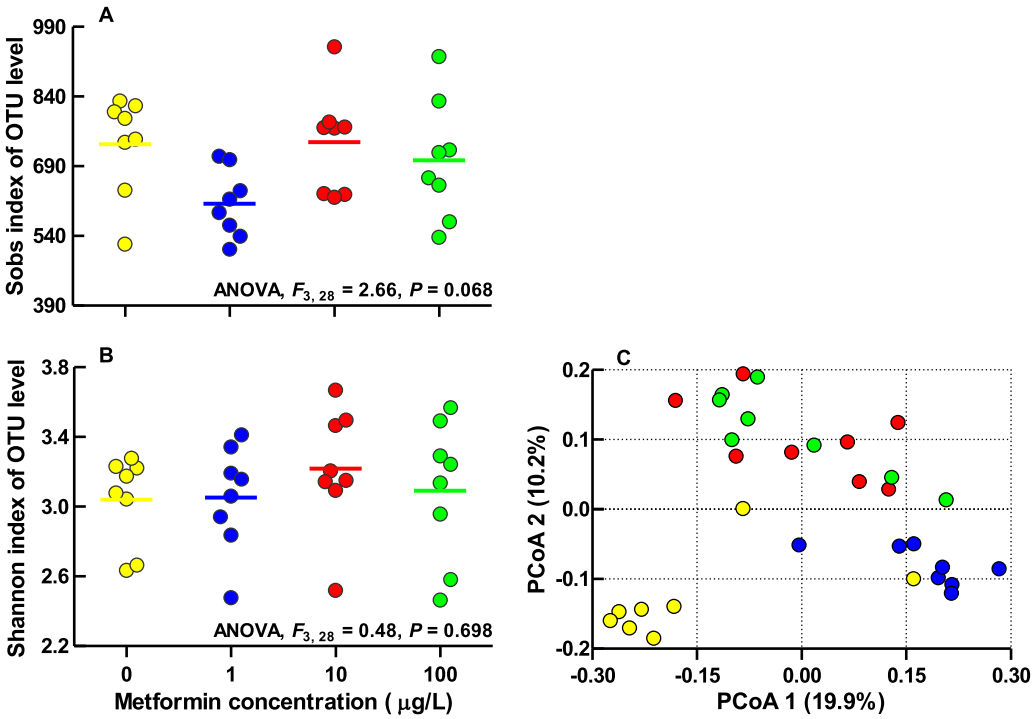

Fig. 2. Results of alpha and beta diversity analysis for intestinal microbiota of *Pelophylax nigromaculatus* tadpoles exposed to 0 (control), 1, 10 and 100 μg/L metformin. No significant differences in the Sobs (A) and Shannon (B) indexes were observed among groups, but distinct separations between the control and treatment groups were revealed by the principal coordinates analysis (C).

intestinal bacterial DNA extraction, amplification, purification and sequencing, as well as data processing, were described previously (Kang et al., 2022). Briefly, after total DNA had been extracted and quantified, the V3-V4 variable region of the bacterial 16 S rDNA gene was amplified through two rounds of PCR using forward (B341F: 5′-CCTACGGGNGGCWGCAG −3′) and reverse primers (B785R: 5′- GACTACHVGGGTATCTAATCC −3′). The product from the first round of PCR was detected using 2.0% agarose gel electrophoresis (2 μL of PCR products), and purified using the AMPure XP Beads (Beckman Coulter, Inc.). A total of 2.5 μL of the first round PCR product was used for reamplification in a second round of PCR, and its products were purified using the QIAquick Gel Extraction Kit (Qiagen GmbH), qualified using the Qubit® Fluorometer (Invitrogen Corporation), and sequenced finally on the Illumina MiSeq® PE300 platform (Illumina Inc.).

Raw paired-end reads were quality-filtered with a minimum length of 100 bp, maximum expected error of 1.0, denoised and finally clustered into operational classification units (OTUs) using a threshold of 97% similarity. Representative sequences of the OTUs were taxonomically annotated against the Silva database, and OTU-level alpha diversity indexes (Sobs and Shannon index) were calculated for each sample. The beta diversity with principal coordinates analysis (PCoA) based on unweighted UniFrac distance was used to compare the microbial community composition among treatments. Functional metagenomes of intestinal bacteria were predicted using PICRUSt 2 based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. One-way analysis of variance (ANOVA) was used to determine differences in alpha diversity indexes among treatments; non-parametric Kruskal-Wallis (and Wilcoxon) tests were used to determine differences in relative abundances of intestinal bacteria and KEGG pathways among treatments. Intestinal microbiomic analysis was completed by Hangzhou Kaitai Biotechnology Co., Ltd. (Hangzhou, China).

2.3. Intestinal metabolomic analysis

The remaining three intestinal content samples from each treatment were used for metabolomic analysis (Fig. 1). Detailed procedures for sample preparation, liquid chromatography-mass spectrometry (LC-MS) analysis and data processing were described previously (Lu et al., 2022). Briefly, after being added to the extraction solution, ground, ultra-sonicated, incubated on ice, and centrifuged at 12,000 rpm, the supernatants of samples were extracted and blow-dried. Dried residues were redissolved with 50% acetonitrile and filtrated before LC-MS detection. Metabolomic profiling of intestinal content was performed on an ACQUITY UPLC system (Waters Acquity, Milford, USA) coupled to a Thermo Q Exactive mass spectrometer (Thermo, San Jose, USA). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used to analyze the metabolomic variations among treatments. Intestinal metabolites were identified by searching the mass spectra against available databases [i.e., Human Metabolome Database (HMDB), MassBank Database]. Non-parametric Kruskal-Wallis tests were used to compare average spectral areas for identified metabolites.

Correlation analysis was applied to evaluate the correlation between significantly-differential intestinal bacterial genera and metabolites. Descriptive statistical values in this paper were presented as mean ± standard error (SE).

3. Results

3.1. Body size

No tadpoles died in each treatment during the exposure. At the end of 30-day exposure, tadpole body mass did not differ significantly among treatment groups (control: 0.100 ± 0.003 g; 1 μg/L: 0.113 ± 0.008 g; 10 μg/L: 0.098 ± 0.005 g; 100 μg/L: 0.107 ± 0.007 g, mixed model ANOVA with experimental treatment as a fixed factor and tank identity as a random factor, $F_{3, 10} = 1.01$, $P = 0.439$) and among experimental tanks ($F_{8, 127} = 1.33$, $P = 0.232$).

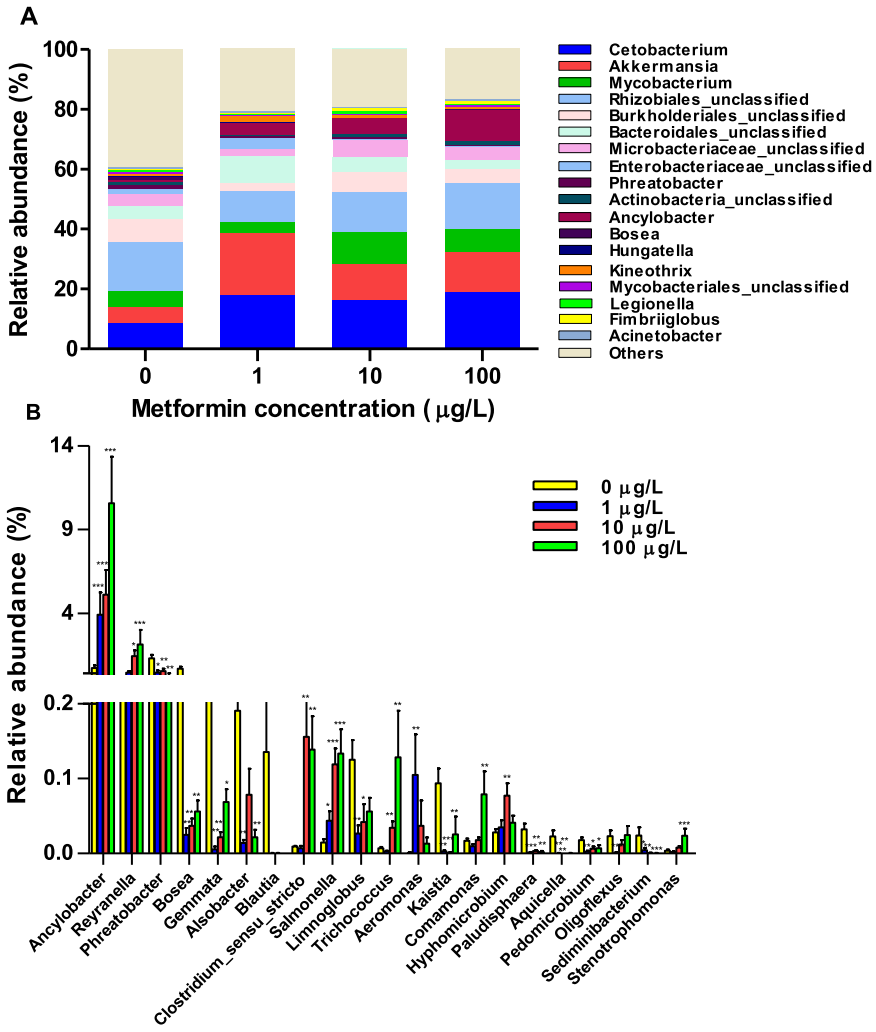

Fig. 3. Relative abundances of intestinal microbiota at the genus level (A), and significantly-changed bacterial genera (B) in *Pelophylax nigromaculatus* tadpoles exposed to 0 (control), 1, 10 and 100 μg/L metformin. Asterisks above the bars in plot B indicated significant differences from the control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.2. Intestinal microbial diversity and composition

A total of 2 430 511 clean reads (approximately 75 953 reads per sample) were obtained from our 32 samples (Table S1). These sequences generated 2528 OTUs. The control, 1, 10, and 100 μg/L MET exposure groups had 1667, 1402, 1762, and 1761 OTUs, respectively. Among them, 885 OTUs were shared among these four groups, and 260, 116, 150, and 177 OTUs were exclusively found in the control, 1, 10, and 100 μg/L MET exposure groups, respectively (Fig. S1).

The richness (based on the Sobs index, Fig. 2A) and diversity (based on the Shannon index, Fig. 2B) of the intestinal bacterial community did not differ significantly among groups. However, the analysis of beta diversity revealed that the intestinal bacterial community in the control group was distinct from those in the exposure groups (PCoA based on unweighted UniFrac distance matrices, Fig. 2C).

Further taxonomic analysis showed that the composition of tadpole intestinal microbiota changed significantly following MET exposure (Fig. 3A). For example, at the genus level, the abundances of some identified bacterial genera [e.g., *Ancylobacter* (belonging to phylum Proteobacteria, family Hyphomicrobiaceae), *Reyranella* (Reyranellaceae), *Salmonella* (Enterobacteriaceae), *Comamonas* (Comamonadaceae), *Stenotrophomonas* (Xanthomonadaceae), *Clostridium_sensu_stricto* (Firmicutes, Clostridiaceae), and *Trichococcus* (Carnobacteriaceae)] increased significantly, while those of some genera [e.g., *Phreatobacter* (Proteobacteria, Phreatobacteraceae), *Bosea* (Boseaceae), *Alsobacter* (Alsobacteraceae), Blautia (Firmicutes, Lachnospiraceae), *Kaistia* (Kaistiaceae), *Gemmata* and *Limnoglobus* (Planctomycetes, Gemmataceae)] decreased in MET-exposed tadpoles (Fig. 3B). Despite accounting for a small proportion (< 0.001%), some identified bacterial genera were found only in the control or low-concentration (1 μg/L) exposed group [e.g., *Prevotella* (Bacteroidetes, Prevotellaceae), *Anaerosporobacter* (Firmicutes, Lachnospiraceae), *Fimbriimonas* (Armatimonadetes, Fimbriimonadaceae)], while some genera were found only in higher concentration exposed groups [e.g., *Duganella* (Proteobacteria, Oxalobacteraceae), *Xanthomonas* (Xanthomonadaceae), *Aquirufa* (Bacteroidetes, Cytophagaceae), Lacticaseibacillus (Firmicutes, Lactobacillaceae)].

PICRUSt 2 analysis predicting functional pathways of intestinal bacteria identified different KEGG pathways at the top (divided into a few broad functional categories), second (divided into 59 subcategories), and third level (further divided into more than 500 pathway maps), respectively. Metabolism, environmental information processing and genetic information processing were the primary categories at the top level; carbohydrate, amino acid, cofactors and vitamins, energy metabolism, membrane transport, lipid metabolism, replication and repair, and terpenoid and polyketide metabolism were the primary functions at the second level (Fig. S2). The abundances of some KEGG pathways at different hierarchical levels differed significantly among

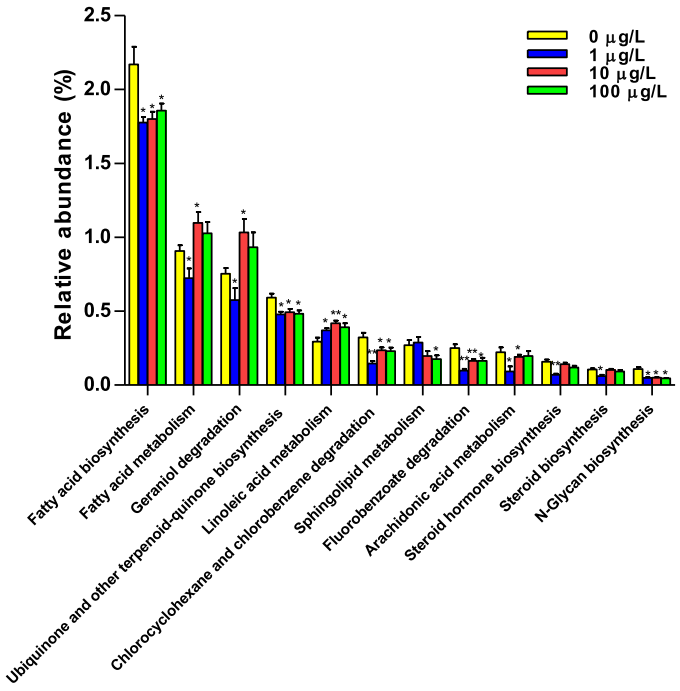

Fig. 4. Relative abundances of predicted functional pathways with significant among-group differences in the intestinal microbiota of *Pelophylax nigromaculatus* tadpoles. Asterisks above the bars indicated significant differences from the control group (* $P < 0.05$, ** $P < 0.01$).

treatment groups. For example, at the third level, the abundances of fatty acid biosynthesis, sphingolipid metabolism and N-Glycan biosynthesis in the control group were higher, but the abundances of fatty and linoleic acid metabolism were lower than those in 10 μg/L- and/or 100 μg/L-exposed groups (Fig. 4).

3.3. Intestinal metabolomic profiles

PCA and PLS-DA on intestinal metabolite data revealed obvious differences between groups (Fig. 5). The first two extracted components from PCA or PLS-DA explained 42.3% and 36.9% of the variation of metabolite data in the negative ion mode, and 42.1% and 32.2% in the positive ion mode, respectively (Fig. 5). By searching and comparing the information from available databases, 16.68% of the selected metabolites could be identified. Some identified metabolites changed significantly following MET exposure. The levels of some intestinal amino acids appeared to decrease (e.g., tyrosine, leucine) or increase (e.g., glutamate) in MET-exposed tadpoles. Similarly, some metabolites were found to decrease (e.g., choline, cholesterol, methionine S-oxide, succinate), while others increased (e.g., deoxyguanosine) (Fig. 6).

The correlation analysis showed the potential links between differential intestinal bacteria and metabolites (Fig. 7). Some microbial members revealed multiple metabolite associations. For example, the abundances of bacterial genera *Paludisphaera*, *Aquicella*, *Bosea* and *Blautia* were positively correlated with the levels of tyrosine, cholesterol, succinate, etc., while that of *Ancylobacter* was negatively correlated with the levels of ribulose, aminophenylbutyric acid, etc. The abundance of *Comamonas*, *Clostridium_sensu_stricto* and *Aeromonas* was solely correlated with the level of glutamate, choline, or methoxamine, respectively (Fig. 7).

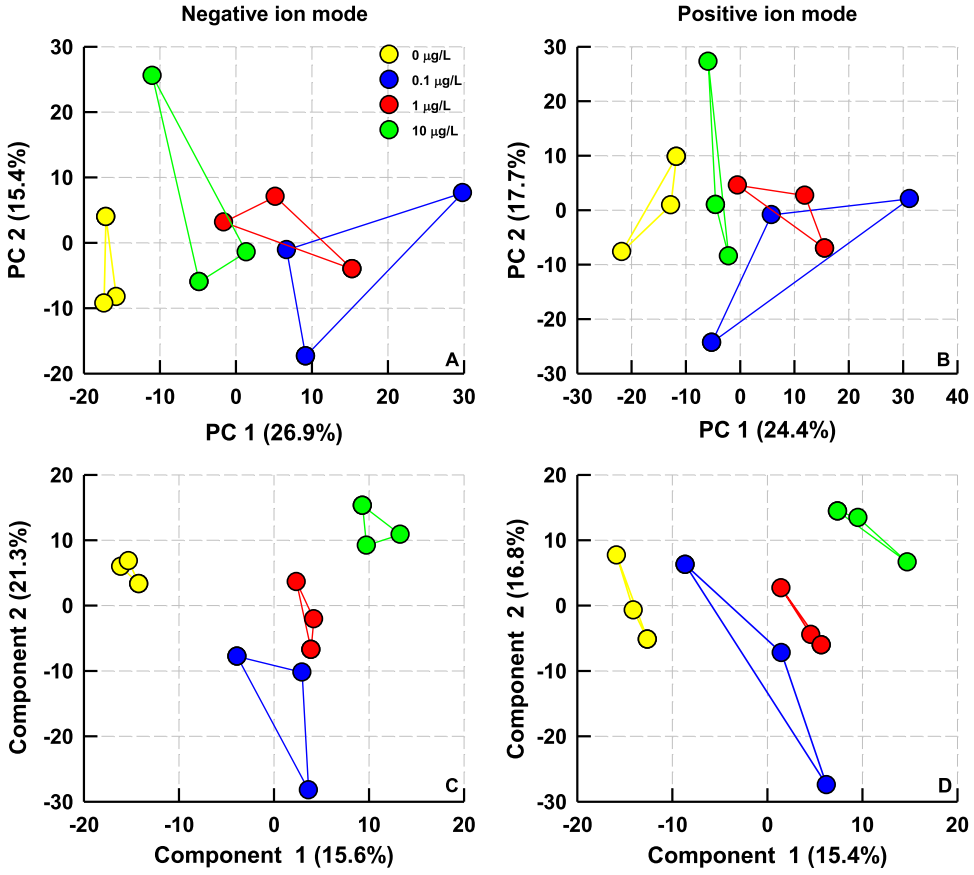

Fig. 5. Results of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) for intestinal metabolite profiles in *Pelophylax nigromaculatus* tadpoles exposed to 0 (control), 1, 10 and 100 μg/L metformin in the negative and positive ion mode, respectively. Obvious among-group differences were revealed by these two multivariate analyses.

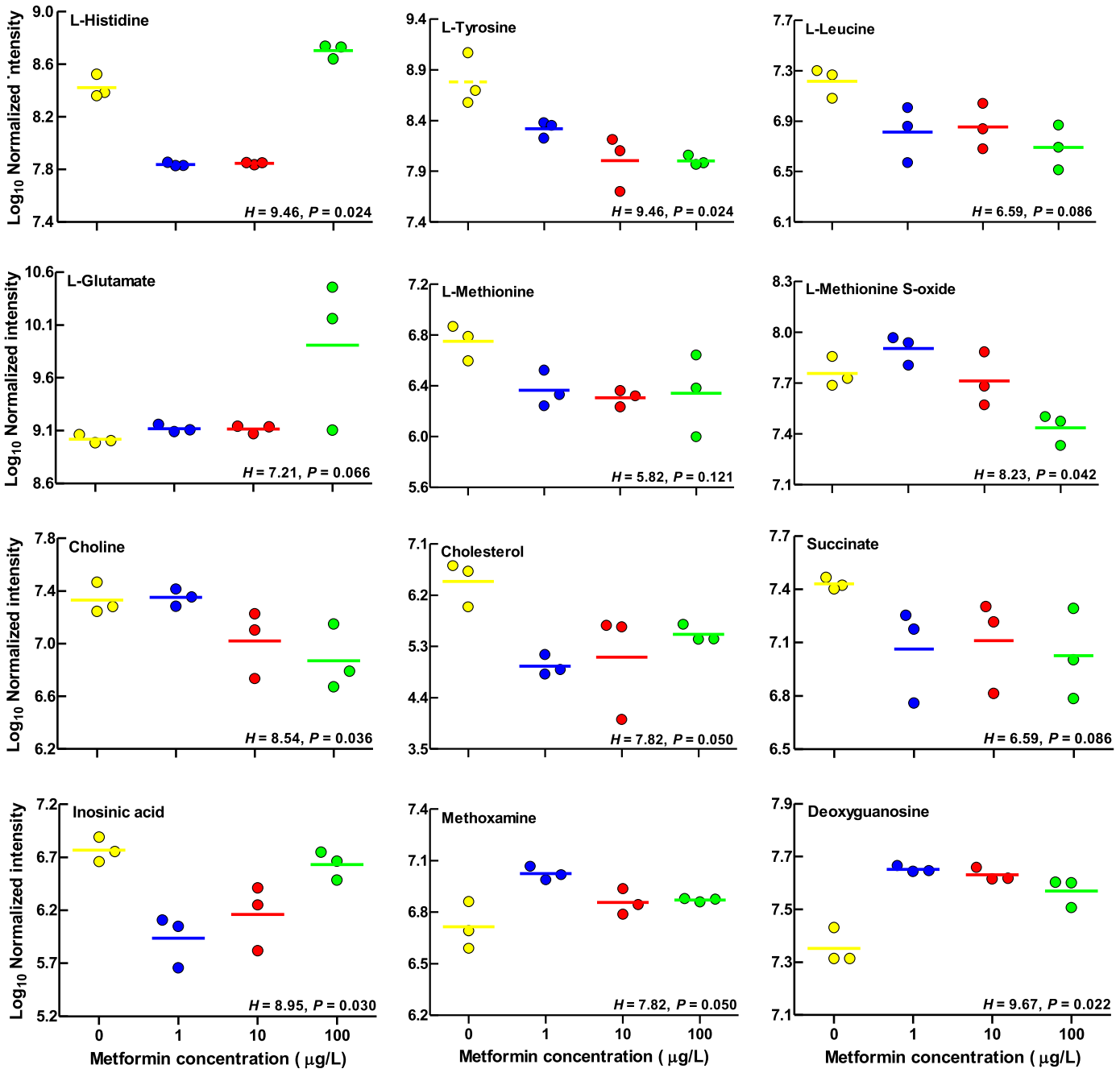

Fig. 6. Several identified intestinal metabolites in *Pelophylax nigromaculatus* tadpoles exposed to 0 (control), 1, 10 and 100 μg/L metformin. Among-group differences in the spectral area for these identified metabolites were tested using non-parametric Kruskal-Wallis tests.

4. Discussion

The issue of potential impacts of pharmaceutical residues on non-target wildlife in water bodies is receiving increasing attention in the field of aquatic toxicology (Rudd et al., 2014; Hernández-Tenorio et al., 2022). Multiple toxicological endpoints related to the antidiabetic pharmaceutical-MET have been assessed in some fish species (e.g., Jacob et al., 2018; Lee et al., 2019; Barros et al., 2022; Elizalde-Velázquez et al., 2022). However, only limited toxicological data are available for other non-fish species, such as amphibians (Melvin et al., 2017). Against this background, this study investigated several physiological changes in MET-exposed *P. nigromaculatus* tadpoles. Although correlated responses in growth and survival traits of tadpoles to MET exposure were not evident, some potential adverse impacts of MET in amphibian larvae might be revealed by changes in intestinal microbiota composition and intestinal metabolites. No significant changes in the alpha diversity of intestinal microbiota were found in MET-exposed tadpoles, which was consistent with the results from two fish species, *Salmo trutta* (exposed to MET at concentrations up to 1000 μg/L, Rogall et al., 2020) and *Pimephales promelas* (exposed to MET at concentrations up to 269 μg/L, Parrott et al., 2022). The alpha diversity indexes could not actually show the differences in the abundance of intestinal bacteria at different taxonomic levels.

Further comparison analyses of intestinal microbial compositions among different MET treatments were performed, indicating that notable changes in microbial composition (especially at lower taxonomic levels) of tadpoles would occur following MET exposure. Proteobacteria, which includes a wide variety of pathogens, was the most

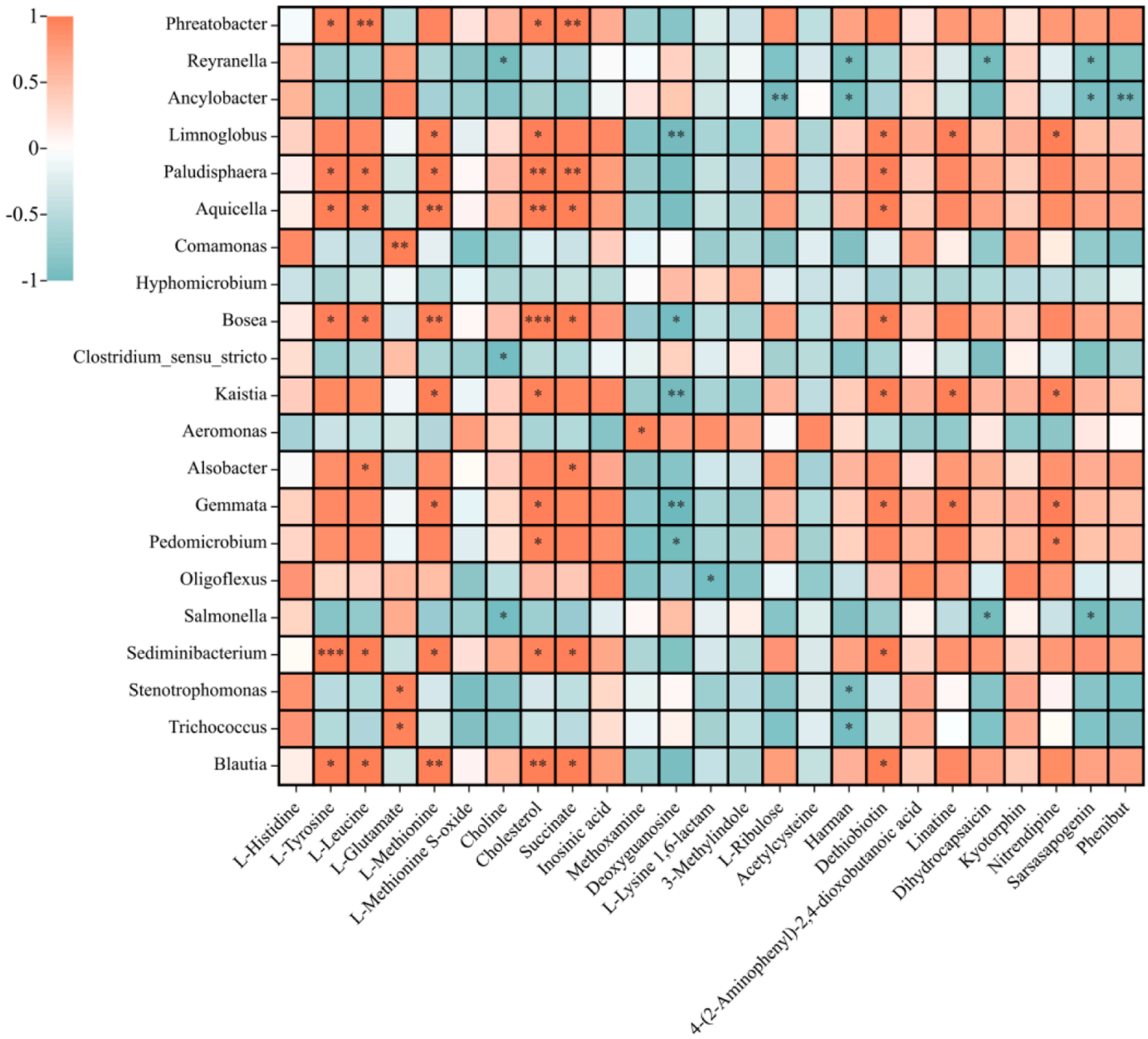

Fig. 7. Results of the correlation analysis between significantly-changed intestinal bacterial genera and metabolites. Asterisks in the grids indicated significant correlations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

abundant bacterial phylum in *P. nigromaculatus* tadpoles. Some pathogenic bacterial genera belonging to Proteobacteria, such as *Salmonella* (common pathogens causing gastrointestinal disease), *Comamonas* and *Stenotrophomonas* (opportunistic pathogens), together with the bacterial genus *Trichococcus* belonging to Firmicutes, were significantly increased in MET-exposed tadpoles, which might have resulted into an unhealthy intestinal state (Ringø et al., 2010). The bacterial genus *Clostridium_sensu_stricto* can play an important role in impacting host metabolism and immunity, but several members of this genus also belong to opportunistic pathogenic bacteria (Burgos et al., 2018). Increased relative abundance of *Clostridium_sensu_stricto* in MET-exposed tadpoles might not necessarily be beneficial for their health. Conversely, some beneficial bacterial genera, such as *Blautia* (mediating beneficial anti-inflammatory effects), *Prevotella* (decomposing polysaccharides and regulating intestinal immune function), were significantly decreased in MET-exposed tadpoles, which might also be associated with intestinal mucosal barrier function damage and inflammation (Evariste et al., 2019; Shintouo et al., 2020). Overall, intestinal microbiota dysbiosis (an increase in pathogenic bacteria and a decrease in beneficial bacteria) was shown to occur and might eventually lead to unhealthy effects in MET-exposed animals.

The changes in the abundance of specific intestinal microbial taxa would affect host metabolism (Lamichhane et al., 2018). Previous studies evidenced that exposure to MET, even at environmentally relevant concentrations, would disrupt multiple metabolic pathways, including energy, lipid, and amino acid metabolism, as well as steroid hormone biosynthesis, in fish (Niemuth and Klaper, 2018; Ussery et al., 2018; Lee et al., 2019; Barros et al., 2022). The functional prediction of intestinal microbiota profiles suggested the potential impacts of microbial changes caused by MET exposure on certain metabolic pathways. For example, the predicted relative abundance of the lipid acid biosynthesis pathway was significantly reduced in MET-exposed tadpoles. It has been well-documented that, besides hepatic gluconeogenesis, MET can inhibit the biosynthesis of lipids in mammalian species

(Madsen et al., 2015). Accordingly, a similar physiological impact might also occur in aquatic species, such as fish (Zhou et al., 2019). The metabolism of other lipids, such as sphingolipids that are produced by *Bacteroides* bacteria, was also affected by MET exposure. Sphingolipids can play an important role in maintaining intestinal microbial homeostasis, and their lower level might increase the risk of inflammation (Brown et al., 2019). Moreover, as a potential endocrine disrupting compound, MET is believed to affect steroid hormones and steroid biosynthesis (Niemuth and Klaper, 2018; Barros et al., 2022). However, lower relative abundances of metabolic pathways of steroid hormone and steroid biosynthesis were only shown in 1 μg/L (not in higher concentrations) MET-exposed tadpoles.

Furthermore, intestinal metabolites were investigated using LC-MS analysis, and multivariate statistics of metabolomic profiles revealed clear separations among different treatment groups, indicating metabolic changes caused by MET exposure. MET-induced metabolic perturbations revealed by metabolomic profiling have also been reported in fish and amphibian species (Melvin et al., 2017; Ussery et al., 2018). These studies showed that several metabolites associated with cellular energetics, cell proliferation/growth, energy and amino acid catabolism were significantly changed (Melvin et al., 2017; Ussery et al., 2018). Some studies have suggested that aromatic (such as tyrosine) and branched chain amino acids (such as leucine) can be used as biomarkers for responses to medical therapy of type 2 diabetes (Walford et al., 2013). Following treatment with pharmaceuticals having hyperglycemic effects (e.g., glipizide and berberine), the levels of aromatic and branched chain amino acids were shown to decrease (Walford et al., 2013; Yao et al., 2020). Consistent with these findings, similar downward trends in intestinal tyrosine and leucine levels were observable in MET-exposed tadpoles. Choline is an essential nutrient for animal health, and its deficiency would influence intestinal function, amino acid absorption, and lipid metabolism, and thus reduce the growth performance of aquatic organisms, such as fish (Yuan et al., 2020). Decreased intestinal choline level in MET-exposed tadpoles might lead to a similar outcome in terms of growth performance. However, it should be noted that the measurement of mass change over a period of only 30 days here was insufficient to evaluate the long-term impact on tadpole growth, and further investigation (upon metamorphosis, or during juvenile and adulthood) is required. Moreover, reduced choline level possibly resulted from its excessive consumption by intestinal bacteria (which would increase the production of harmful trimethylamine, Chittim et al., 2019) and the decrease of endogenous choline synthesis (which requires S-adenosyl methionine-SAM as the methyl donor, Visioli et al., 1998). Despite the lack of a statistically significant difference, an observed downward trend in a precursor for the synthesis of SAM, methionine, together with a lowered level of methionine S-oxide (which can be reduced to methionine under the catalysis of methionine sulfoxide reductase), could predictably influence choline synthesis (Espe et al., 2017). The inosinic acid (IMP) level exhibited a non-monotonic dose-response relationship for exposure to MET, with a decrease at lower concentrations and an increase to a level similar to that in the control group at a higher concentration of MET. IMP has been documented to improve the antioxidative ability of fish (Hossain et al., 2016). The alteration of IMP in the intestine of MET-exposed tadpoles might be a protective response against oxidative stress (Hossain et al., 2016; de Cruz et al., 2020).

5. Conclusion

This study investigated the impacts of exposure to an antidiabetic pharmaceutical on body size, intestinal microbial diversity and composition, and metabolites in amphibian larvae. As reported in other aquatic species (Rogall et al., 2020; Parrott et al., 2022), no evident difference in body size or intestinal microbial diversity was found in tadpoles exposed to environmentally relevant concentrations of MET for 30 days. However, the intestinal microbial composition was shown to change significantly. Some common or opportunistic pathogenic bacteria were found to increase, while some beneficial bacteria decreased in the intestine of MET-exposed tadpoles. This indicated that MET exposure would cause intestinal microbiota dysbiosis, thereby influencing the health status of animals. Correspondingly, the levels of some identified intestinal metabolites potentially associated with growth and immune performance also changed significantly following MET exposure. Considering these alterations in intestinal microbial composition and metabolites together, our results suggest that exposure to antidiabetic pharmaceuticals, even at environmentally relevant concentrations, would have some adverse effects on non-target aquatic wildlife.

CRediT authorship contribution statement

Guang-Li Fu: Investigation, Writing - original draft. **Qin-Yuan Meng**: Investigation, Software. **Yu Chen:** Data curation. **Jin-Zhao Xin:** Methodology. **Jia-Hui Liu**: Data curation, Validation. **Wei Dang**: 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115617.

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