Eco-Environment & Health 5 (2026) 100231

Contents lists available at ScienceDirect

Eco-Environment & Health

journal homepage: www.journals.elsevier.com/eco-environment-and-health

Original Research Article

Metabolomic and microbial responses of multilevel aquatic organisms to antibiotics in freshwater microcosm: The uniformity and specificity

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ARTICLE INFO

Keywords:
Micropollutants
Metabolomics
Microbiomics
Dysregulation
Self-coordination

ABSTRACT

The widespread presence of antibiotics in aquatic environments raises concerns about their ecological impacts. However, the molecular-level effects of antibiotics and the underlying mechanisms, particularly the responses across aquatic species, remain unclear. We established a freshwater microcosm including duckweeds (*Salvinia natans*), snails (*Cipangopaludina cathayensis*), and fish (*Danio rerio*) to investigate their uniform and specific responses to antibiotics (sulfamethoxazole, ciprofloxacin, oxytetracycline, and azithromycin), each at 1, 10, and 100 μg/L for 45 days. Antibiotic exposure diminished chlorophyll content in duckweeds, increased soluble sugar levels, elevated triglyceride levels in snails, and raised total bile acid concentrations in fish. Metabolomic analysis revealed that both duckweeds and fish tended to store energy to defend against antibiotic-induced stress, but through different pathways. Duckweeds accumulated sugar metabolites and downregulated antioxidants, while fish consumed primary sugars and converted them into lipid metabolites. Microbiome analysis indicated a self-coordination of gut bacteria in both snails and fish exposed to 1 and 10 μg/L of antibiotics, while dysbiosis occurred in snails at 100 μg/L, marked by increased pernicious bacteria abundance. In contrast, the abundance of probiotic bacteria increased in the fish gut due to microbial resistance to antibiotics, which played a crucial role in bile acid metabolism and positively influenced hepatic lipid metabolism via the gut-liver axis. This study uncovered the uniform and specific defense and dysregulation behaviors of multilevel aquatic organisms in response to antibiotic exposure, providing valuable insights into the selection of molecular-level endpoints for water quality benchmark development to safeguard aquatic life from antibiotic pollution.

1. Introduction

The increasing production of antibiotics has led to subsequent environmental releases and contamination, raising great concern about the associated environmental risks, including the emergence of antibiotic resistance genes (ARGs) [1,2]. Due to the relatively low removal efficiency in wastewater treatment plants (WWTPs), numerous antibiotics are discharged into surface waters through effluent [3,4]. It was estimated that 23% of total emissions of 80 veterinary antibiotics were ultimately released into surface water between 2010 and 2020 [5]. A systematic review across 76 countries identified about 160 antibiotics in global surface waters, among which sulfamethoxazole (SMX), ciprofloxacin (CIP), oxytetracycline (OTC), and azithromycin (AZM) were frequently detected with concentrations ranging from ng/L to μg/L, reaching mg/L levels in some cases [6,7]. For example, SMX showed an average concentration of 2 μg/L with a maximum of 129 μg/L [7]. Current frameworks for assessing antibiotic effects in aquatic environments primarily rely on ecotoxicological tests using 50% effect concentrations (EC_{50}) [8,9], which predominantly focus on lethal or phenotype endpoints and serve as regulatory thresholds [10]. For example, a global survey of 258 rivers revealed widespread occurrence of multiple antibiotics at concentrations exceeding safety thresholds for

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https://doi.org/10.1016/j.eehl.2026.100231
Received 28 July 2025; Received in revised form 26 November 2025; Accepted 11 March 2026
Available online 12 March 2026
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aquatic organisms based on the growth inhibition of algae and immobilization of invertebrates [11]. However, growing evidence highlights the limitations of lethal endpoints, as they overlook sublethal molecular effects of pollutants occurring at environmentally relevant concentrations [12]. This oversight is particularly critical for antibiotics, which result in chronic exposure of aquatic organisms due to continuous environmental discharge and persistence, posing potential long-term risks to ecosystem health that are not captured by traditional acute toxicity assessments [13]. Consequently, there is an increasing recognition that elucidating the sublethal molecular mechanisms of pollutants is indispensable for advancing ecological risk assessment frameworks.

Ecotoxicological studies suggest that sublethal concentrations of antibiotics can directly induce tissue damage and oxidative stress in aquatic organisms, e.g., exposure to 0.3 μg/L of SMX induced oxidative stress, immunotoxicity, and inflammatory injury in grass carp (*Ctenopharyngodon idella*) after 42 days [14,15] and inflammatory injury in carp kidneys after 30 days [16], and 7-d exposure to 1 mg/L of CIP induced malondialdehyde increase in duckweeds [17]. Beyond direct damage, antibiotics have been reported to dysregulate the homeostatic balance of gut bacteria, which may indirectly increase disease susceptibility by impairing key microbial functions such as fat metabolism and immune system maintenance [18]. Previous studies have demonstrated that antibiotic exposure altered gut microbiomes of aquatic organisms, including reduced microbial diversity and bacterial shift in zebrafish gut exposed to 100 μg/L of enrofloxacin [19]. While these studies underscore the multifaceted nature of antibiotic toxicity, they have primarily relied on phenotypic endpoints or microbiome profiles. A comprehensive, molecular-level understanding of the specific metabolic disruptions and the role of gut microbiota in mediating these effects across different species remains limited.

The EC_{50} values of certain antibiotics differ in multilevel organisms [6,11], but the uniform and specific molecular responses of organisms to antibiotics remain unclear. Cross-species comparisons of the antibiotic mode of action are challenging due to the divergent experimental conditions employed. Although various organisms activate protective mechanisms against oxidative stress induced by environmental challenges [20,21], their responses might differ, driven by the inherent biological characteristics. Similarly, while pollutant exposure generally alters gut microbiota composition across species, the affected bacterial taxa and physiological consequences vary [19,21,22]. Moreover, gut bacteria critically regulate liver function, including lipid metabolism via the gut-liver axis [23], yet their role in antibiotic-induced metabolic changes remains poorly understood. In this context, we hypothesized that antibiotic exposure causes direct and gut bacteria-mediated responses in aquatic organisms, featuring both uniform and species-specific metabolomic and microbial alterations.

In this study, we established a freshwater microcosm containing duckweed, freshwater snail, and zebrafish as representatives of floating plants, benthos, and fish, respectively. Four antibiotics, i.e., SMX, CIP, OTC, and AZM, which belong to sulfonamide, fluoroquinolone, tetracycline, and macrolide, respectively, and are frequently detected in surface water [6,7], were introduced into microcosms to investigate: (1) the regulation of metabolomics in aquatic plants and animals exposed to antibiotics, (2) the microbial response of gut bacteria and ARGs within aquatic animals, and (3) the uniformity and specificity in multilevel aquatic plants and animals in responses to antibiotics. By integrating metabolomic and microbiome analyses, this study aimed to address the direct and indirect molecular effects of antibiotics on multilevel aquatic organisms after long-term exposure, and advance fundamental understanding of the complex response mechanisms of multilevel aquatic organisms to antibiotic exposure. The identified metabolic and microbial effects may be incorporated into molecular-level endpoints to support water quality benchmark development for antibiotic pollution control.

2. Materials and methods

2.1. Chemicals

SMX and CIP (99% of purity) were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China). OTC and AZM (99% of purity) were purchased from Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). Other chemicals of chromatographic or analytical grade were from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China), Macklin Reagent Co. Ltd. (Shanghai, China), and Aladdin Reagent Co. Ltd. (Shanghai, China).

2.2. Exposure of multilevel aquatic organisms to antibiotics in freshwater microcosms

Three aquatic plants and animals, duckweeds (*Salvinia natans*), freshwater snails (*Cipangopaludina cathayensis*), and zebrafish (*Danio rerio*), were separately cultivated for three months. The acquisition and cultivation of these organisms are shown in Text S1. Then five duckweeds (0.15 ± 0.03 g), six snails (2.94 ± 0.74 g), and six fish (0.84 ± 0.21 g) were pre-incubated together for two weeks in 1 L of tap water, which had been aerated for 48 h, in flow-through microcosm systems (12 cm × 12 cm × 12 cm of glass tank, $n = 12$) at 25 °C under a 16 h light/8 h dark photoperiod. Organism population sizes were determined to ensure sufficient biological material for subsequent analyses. It should be noted that the microcosm was designed not to simulate complex ecosystem-level interactions but to ensure that all organisms were exposed to the same water conditions, such as nutrient levels and microbial communities therein, thereby controlling environmental variability that might influence biological response. Pre-experiments confirmed the absence of predator-prey interactions among the investigated species. Fresh aerated tap water was continuously pumped into the tank with a hydraulic retention time of 24 h. The tanks were washed twice per week to remove feces, during which the organisms were transferred to extra clean tanks. Snails and fish were fed twice daily with concentrated algae (*Chlorella pyrenoidosa*) and fish fodder, respectively.

After pre-incubation, the aerated tap water was replaced with a mixture of water collected from the Yangtze River (32°10′4″N, 118°56′11″E) and wastewater effluent from a WWTP in Nanjing (40:1, v/v), to simulate the aquatic environment of river water receiving WWTP effluent [24]. The twelve flow-through freshwater microcosms were randomly assigned into four groups and exposed to the river water spiked with a mixture of four antibiotics (SMX, CIP, OTC, and AZM), with each antibiotic in the mixture maintained at the same concentration of either 0 (control), 1, 10, or 100 μg/L ($n = 3$) throughout the incubation. The exposure concentrations were chosen to encompass a range of environmental scenarios, representing normal environmentally relevant levels, moderately elevated levels, and potential worst-case exposure conditions in hotspot areas (Table S1). The cleaning and feeding procedures were identical to those used for the pre-incubation. Although these procedures introduced external nutrients and microorganisms into microcosms or periodically removed a portion of the established microbial community, they were strictly uniform across all experimental groups, ensuring that the observed biological responses were attributable to antibiotic exposure. The influent and effluent samples (about 250 mL) of the four freshwater microcosms were collected every week for analysis of water quality and antibiotic contents. After 45 days of incubation, the duckweeds, snails, and fish were collected for phenotypic and omics analyses. The exposure time was set according to previous studies examining the phenotypic responses of duckweeds and fish to antibiotics, where exposure durations ranged from 1 to 150 days with an average of 43 days (Table S2). Furthermore, we employed mature organisms to minimize confounding effects from

rapid growth and development. A blank group without the plants and animals ($n = 3$) was run simultaneously in the same flow-through system to evaluate the hydrolysis of 100 μg/L antibiotics under culture conditions.

2.3. Water quality and antibiotic content analyses

After sampling of influents and effluents, the pH and dissolved oxygen (DO) were measured immediately by a pH meter (FiveEasy Plus, Mettler Toledo, Switzerland) and a DO meter (SmartAR8010, SMART SENSOR, China), respectively. The concentration of chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) was measured using national standard methods [25]. To determine the antibiotic concentrations, 50 mL of the samples were filtered by 0.22-μm glass fiber membranes, then concentrated and purified by solid phase extraction, and measured using high-performance liquid chromatography (HPLC; Agilent 1200, USA) connected to a triple quadrupole mass spectrometer (MS/MS; API4000, AB SCIEX, USA) (HPLC-MS/MS). More details on the sample preparation, detection, quality assurance, and quality control are shown in Text S2, Fig. S1, and Table S3.

2.4. Phenotypic analysis

The wet weights of duckweeds, snails, and fish were determined as biomass before and after a 45-day incubation. No mortality occurred in any group, and the removal of snail offspring during cleaning did not significantly change their population across treatments. At the end of the incubation, the duckweeds were frozen and ground in liquid N_2. The snails and fish were dissected and separately frozen in liquid N_2. The photosynthetic pigments, total phenolic, and soluble sugars in duckweeds were extracted and quantified as described in Text S3. The contents of triglycerides in muscles and total bile acids in the guts of snails and fish were also determined (see more details in Text S4).

2.5. Metabolomic analysis of duckweeds and fish

For metabolomic analysis, 60 mg of the ground duckweeds were ultrasonically extracted with 600 μL of a methanol/water mixture (1:1, v/v) containing the internal standard 2-chloro-L-phenylalanine (4 μg/mL) for 30 min. Then 150 μL of chloroform was added and sonicated for 30 min. After centrifugation for 10 min (14,000×*g*, 4 °C), 300 μL of the supernatant was freeze-dried, followed by the addition of methoxylamine hydrochloride solution (80 μL, 15 mg/mL in pyridine). The mixture was vortexed for 2 min and incubated at 37 °C for 90 min. Afterwards, 80 μL of N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (with 1% trimethylchlorosilane) and 20 μL of *n*-hexane were added to the mixture. The mixture was vortexed for 2 min and heated at 70 °C for 60 min to allow the silylation reaction. The derivatized mixture was cooled down at room temperature for 30 min before analysis by gas chromatography-mass spectrometry (Text S5). Fish were selected as the representative aquatic animals to determine the response of hepatic metabolism to antibiotic exposure. The liver tissues of fish collected from the same microcosm were pooled as one sample due to the limited biomass. After weighing, the whole liver tissues (1.7 ± 0.7 mg) were transferred into a 1.5 mL centrifuge tube with two small steel balls, followed by the addition of 200 μL of methanol/water mixture (4/1, v/v) containing the internal standard 2-chloro-L-phenylalanine (4 μg/mL). The mixture was ground for 2 min, and the following extraction protocol of metabolites was the same as that of duckweeds.

2.6. Analyses of bacteria and ARGs in the guts of snails and fish

The composition and function of bacterial communities in the guts of snails and fish were investigated using high-throughput sequencing of bacterial 16S rRNA genes. The gut microbiota composition was selected as the primary endpoint rather than the water-phase microbial community because the latter was subject to frequent perturbation from dynamic influent and twice-weekly cleaning. As with the fish, the guts of snails in each system were dissected out and collected as one sample at day 45. The DNA of gut bacteria was extracted using a DNA extraction kit (MoBio Laboratories, Powersoil, USA) according to the manufacturer's instructions. DNA concentration and purity were determined using NanoDrop One (Thermo Scientific, USA) and 1% agarose gel. The 16S V3−V4 region of the genes was amplified by a polymerase chain reaction (PCR) system using primers 515F and 806R, and then sequenced on Illumina Hiseq2500 platform (Illumina, San Diego, USA) as described in Text S6.

To quantify relative ARG abundance in the guts, the extracted DNA samples were amplified through high-throughput quantitative PCR on the PCR system (QuantStudio™12K; Thermo Scientific, USA). Relative copy numbers of eight ARGs (*sul1*, *sul2*, *qnrB*, *qnrS*, *tetA*, *tetB*, *ermA*, and *ermB*) and one mobile genetic element (MGE; *intl1*), which were prevalent resistance determinants associated with the four antibiotic classes [26–28], were determined based on threshold cycle (C_T), followed by normalization to the housekeeping gene 16S rRNA (Text S7). The PCR primers and procedures are provided in Tables S4 and S5, respectively.

2.7. Statistical analyses

A one-way ANOVA (Tukey test, $p < 0.05$) was performed to analyze the significant differences in the content of water quality, antibiotics, ARG abundance, and phenotype metrics. Supervised partial least squares discriminant analysis (PLS-DA) and biological pathway analysis based on the metabolome of duckweeds and fish liver were conducted using MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/). Differential metabolites induced by antibiotic exposure were screened according to PLS-DA component 1 (variable importance in projection; VIP >1) and a Student's *t*-test ($p < 0.05$) according to a previous study [29]. The Shannon index was selected to evaluate the α-diversity of the bacterial community, and principal coordinate analysis (PCoA) was used to evaluate the β-diversity. Dissimilarity analysis was used to compare the differences of bacterial communities in different groups based on Bray-Curtis distances using the corrplot package in R software [30]. The operational taxonomic units (OTUs) with relative abundance of >0.5% and discriminated fish liver metabolites (VIP >1.5 and $p < 0.05$) were selected to construct the co-occurrence network in Cytoscape 3.9.1 with a screening limit of correlation coefficient (r) > 0.9 and $p < 0.01$.

3. Results and discussion

3.1. Water quality indicators and antibiotics in microcosms

During the 45 days of incubation, pH and concentrations of DO, TN, and TP in the wastewater significantly altered after passing through the microcosms, possibly owing to the metabolism and excretion of organisms (Fig. S2). However, the values of these parameters in the effluent did not differ significantly between the microcosms with or without the presence of antibiotics, indicating that mixed antibiotic exposure did not affect the water quality in the freshwater microcosms.

Trace amounts of antibiotics (0.1–0.4 μg/L) were detected in the river water (Fig. 1). Concentrations of all four antibiotics in the effluents were significantly lower than those in the influents (10 and 100 μg/L groups). We estimated the accumulation of the antibiotics in the organisms using bioconcentration factors reported in previous studies (Table S6), because it was not possible to quantify the antibiotics in the target organisms due to the limited biomass. The estimated antibiotic contents in organisms, e.g., 47 μg/kg of SMX and 0.1 μg/kg of OTC, were comparable to those in real aquatic environments (Table S7). This low bioaccumulation (<0.002%) indicated its insignificant contribution to the decrease of antibiotics in the effluents. In addition, hydrolysis of antibiotics was observed to be negligible in the blank group except for OTC, which was 25.0% ± 5.3% hydrolyzed (Fig. S3). The considerable

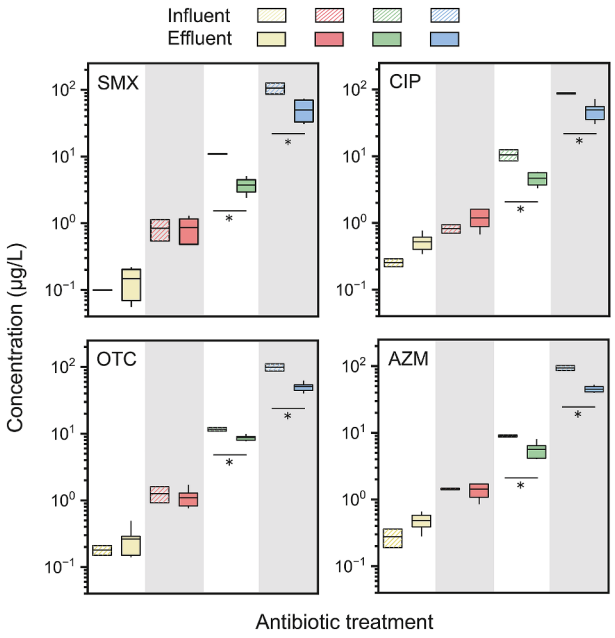


Fig. 1. Concentrations of sulfamethoxazole (SMX), ciprofloxacin (CIP), oxytetracycline (OTC), and azithromycin (AZM) in influents and effluents of the freshwater microcosms ($n = 3$). The initial exposure concentration of individual antibiotics in influents was 0, 1, 10, and 100 μg/L. The asterisk indicates a significant ($p < 0.05$) difference between the concentrations in the influents and effluents.

hydrolysis of OTC was still lower than its decrease in the microcosmic systems (49.1% ± 5.5%) (Fig. 1). Therefore, the decrease of overall antibiotic concentrations in the microcosms was not attributed to hydrolysis, but possibly to other factors such as adsorption on skin and secretions of aquatic organisms, as well as the green algae added as snail food, and degradation by organisms in water. In addition, it should be noted that these estimates from static systems cannot be directly equated to those in our continuous-flow conditions. Future quantification under flow-through exposure conditions is needed to accurately assess antibiotic accumulation in organisms.

3.2. Phenotypic responses of the target organisms to antibiotics

At harvest, the biomass weight of duckweeds increased in the microcosms with or without antibiotic exposure (Fig. S4), consistent with the biomass response of a single species to antibiotic exposure in some of the previous studies (Table S2). Under antibiotic exposure, the contents of chlorophyll *a, b*, and carotenoid in duckweeds significantly decreased in a dose-dependent manner (Fig. S5). Chloroplasts are important targets of antibiotics in plant cells. Unlike previous studies reporting unchanged chlorophyll content in duckweeds exposed to mg/L of antibiotics for ≤7 days (Table S2), our work demonstrated a significant decrease in chlorophyll content when duckweeds were exposed to 100 μg/L of mixed antibiotics. This highlights the chronic damage of duckweeds under antibiotic exposure. Carotenoid, as a non-enzymatic antioxidant, can mitigate the generation of ROS in chloroplasts to protect photosynthetic apparatus [31]. Similar to chlorophyll, the content of carotenoid also decreased under exposure to 100 μg/L of mixed antibiotics. The total phenol content in duckweeds was not changed under antibiotic exposure (Fig. S5). However, we observed a significant increase in the content of total soluble sugars, which function as an energy source and osmoprotectant [20,32]. These findings suggest that duckweeds might alter their energy storage and oxidative stress management strategies under antibiotic stress.

Although antibiotic exposure did not induce notable changes in the biomass of snails and fish (Fig. S4), we observed distinct physiological responses of the animals. Snails significantly increased triglyceride levels under 100 μg/L of antibiotic exposure (Fig. S6). This elevation may result from hepatic regulation of triglyceride synthesis [33]. In contrast to snails and findings of previous studies (Table S2), fish maintained stable triglyceride concentrations but increased gut bile acid content (Fig. S6). Considering that bile acids are known to be metabolized by gut microbiota and are involved in lipid regulation in the liver through the gut–liver axis [34], the observed differences in lipid accumulation between the two species may be attributed to species-specific gut microbiota modulation under antibiotic exposure.

3.3. Metabolomic responses of duckweeds to antibiotics

Plants have developed various physiological, molecular, and metabolic strategies to cope with adverse environmental conditions [35]. To understand the direct effect of antibiotics on cellular homeostasis in duckweeds, we compared the metabolomic responses of duckweeds to the mixture of antibiotics at different concentrations (0, 1, 10, and 100 μg/L). The PLS-DA scores demonstrated a clear distinction of metabolomic composition among the four groups (Fig. S7). According to the VIP score and Student's *t*-test ($p < 0.05$), we screened out 22, 39, and 39 metabolites that were significantly regulated in duckweeds exposed to antibiotics at 1 μg/L, 10 μg/L, and 100 μg/L, respectively, including amino acids, sugars, and sugar alcohol, organic acids, and others (Fig. 2).

Amino acids act as building blocks for several biosynthesis pathways and play pivotal roles in plant stress response [36,37]. We found that several amino acids (e.g., glycine and phenylalanine) were upregulated, which are related to stress defense [38,39] (Fig. 2). L-Glutamic acid, an important precursor of leaf chlorophyll and related to the primary nitrogen metabolism [40], was downregulated under antibiotic exposure, indicating the inhibitory effects of antibiotics on the nitrogen cycle and chlorophyll synthesis in duckweeds.

Consistent with the increasing trend of total soluble sugars (Fig. S5), all the sugar and sugar alcohol metabolites in duckweeds were upregulated under exposure to antibiotics (Fig. 2), such as glucose as an energy source and *myo*-inositol and lactitol as osmoprotectants [41,42]. Similarly, upregulation of several organic acids related to stress defense was observed under exposure to 1 and 10 μg/L of antibiotics, such as quinic acid (a common antioxidant in plants) [43] and shikimic acid (a phenolic metabolite associated with defense response in plants) [44]. However, we found a significant downregulation of several organic acids and other metabolites functioning as antioxidants (e.g., 4-aminobutyric acid, arbutin, cadaverine, and 3-pyridinol) in duckweeds exposed to 100 μg/L of antibiotics (Fig. 2c). For example, 4-aminobutyric acid, as a powerful antioxidant to clear intracellular ROS [45], was downregulated by a 0.58-fold change. This phenomenon indicated that high concentrations of antibiotics might disrupt the regulation of antioxidative metabolites within duckweeds, consistent with the decrease in chlorophyll and carotenoid contents (Fig. S5). Taken together, exposure to antibiotics led to reprogramming of metabolites in duckweeds, mainly including the accumulation of sugar metabolites to store energy in response to stress, along with homeostasis imbalance in defense-related metabolism.

3.4. Metabolomic responses of fish liver to antibiotics

Considering that the liver is the primary detoxification organ and exhibits the highest bioaccumulation of antibiotics compared to other organs or tissues of aquatic organisms [46], we focused on the metabolomic responses of fish liver to antibiotics and compared them with those in duckweeds. Also, a distinguishable metabolic pattern was found in fish liver under antibiotic exposure (Fig. S8). According to PLS-DA analysis, exposure to antibiotics at 1 μg/L, 10 μg/L, and 100 μg/L led to the regulation of 25, 23, and 28 metabolites, respectively. In addition to the same categories of sugars found in duckweeds, several lipid

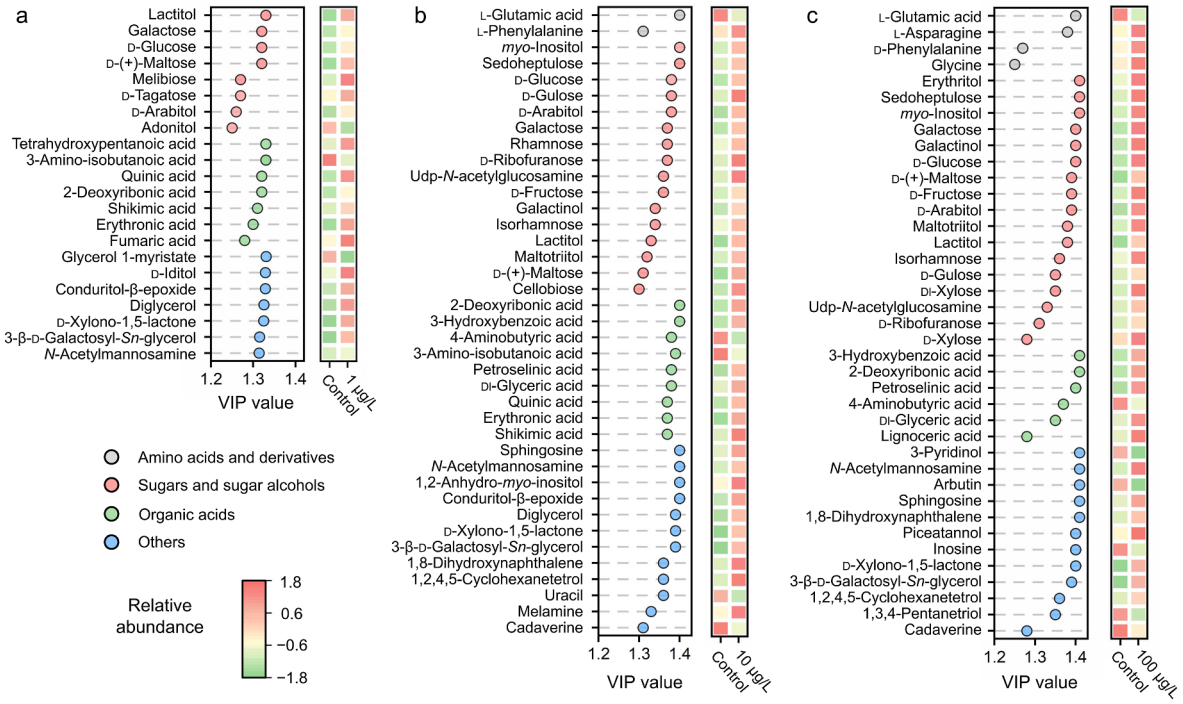


Fig. 2. Variable importance in projection (VIP) score and relative abundance of discriminated metabolites in duckweeds after exposure to mixed antibiotics each at (a) 1 μg/L, (b) 10 μg/L, and (c) 100 μg/L for 45 days. The duckweeds collected from the same microcosm were pooled as one sample (n = 3).

metabolites were screened out in fish liver (Fig. 3a–c).

In contrast to considerable upregulation of sugar metabolites in duckweeds, the content of primary sugar metabolites (e.g., glucose) was reduced, and several secondary sugars (e.g., fructose 1-phosphate and ribofuranose) were accumulated in fish liver, especially in the microcosms exposed to 10 and 100 μg/L of antibiotics (Fig. 3b and c). This alteration in sugar metabolites indicated that antibiotic exposure might induce active carbohydrate metabolism to promote primary sugar decomposition and secondary sugar accumulation in the fish liver. The observed upregulation of adenosine monophosphate and downregulation of hypoxanthine provided further evidence that antibiotic exposure caused disturbances in energy metabolism in fish liver (Fig. 3c), which might promote glycolysis by decomposing adenosine triphosphate into monophosphate to obtain sufficient energy [47,48].

Considering that liver is a key regulator of systemic lipid metabolism [49], the observed glycogen decomposition in fish liver suggests potential concomitant alterations in lipid metabolism, as reported in mice liver exposed to ZnO nanoparticles and imidacloprid [50]. In the microcosms exposed to 10 and 100 μg/L of antibiotics, the screened lipid metabolites [e.g., Mg(16:0/0:0/0:0) and 25-hydroxycholesterol] were all upregulated in fish liver, accompanied by the downregulation of primary sugar metabolites (Fig. 3b and c). Some of the elevated lipid metabolites, including monoacylglycerols [Mg(16:0/0:0/0:0) and Mg (0:0/16:0/0:0)] and glycerol 1-phosphate, are the synthetic precursors of triacylglycerol and have the potential to induce obesity [49]. In addition, dysregulation of various fatty acids (e.g., suberic and eicosadienoic acids, Fig. 3c) also indicated disorder of lipid metabolism in liver. Unlike a recent study reporting that lipid accumulation effects were attributed to antibiotic exposure through increased levels of several specific lipid metabolites, i.e., triglycerides and cholesterol contents [51], our findings provide evidence from a metabolic pattern perspective, emphasizing lipid accumulation accompanied by glycogen decomposition. Given that in our study the accumulation levels of antibiotics in the target organisms align with those in real aquatic environments (Table S7), the effects of antibiotics on non-target aquatic organisms, particularly their tendency to accumulate lipids for energy storage, warrant more attention.

Notably, among the lipid metabolites, the content of 25-hydroxycholesterol was upregulated by an 11-fold change in fish liver exposed to 100 μg/L of antibiotics (Figs. 3c and S9a). Cholesterol can be synthesized into primary bile acids by host hepatocytes, and then modified into secondary bile acids through gut bacteria (Fig. S9b), which are essential for regulating lipid metabolism in livers [52]. In response, two secondary bile acids, ursodeoxycholic acid (3.8- and 4.8-fold change under 10 and 100 μg/L of antibiotic exposure, respectively) and deoxycholic acid (0.6-fold change under 100 μg/L of antibiotic exposure), were indeed significantly regulated (Figs. 3c and S9a). These results aligned with the observed increase in bile acid content in guts (Fig. S6), and suggested that antibiotic exposure may affect the community of gut bacteria and further regulate the liver lipid homeostasis via the gut-liver axis.

In total, both duckweeds and fish tended to regulate their metabolites to store energy as a direct response to antibiotic exposure (Fig. 3d). However, the metabolic response patterns were different between these two freshwater organisms. While sugar metabolites were significantly upregulated with downregulation of antioxidants in duckweeds, primary sugar metabolites in fish liver broke down into secondary sugars and led to the accumulation of lipids. These findings supported our hypothesis that antibiotic exposure induces both uniform and species-specific metabolic responses in multilevel aquatic organisms, emphasizing the necessity of applying sensitive omics tools to comprehensively assess molecular-level effects of antibiotics.

3.5. *Responses of gut microbiota of snails and fish to antibiotics*

To address the hypothesis that antibiotic exposure indirectly influences host metabolism by altering the gut bacterial community, we compared the bacterial composition in the guts of snails with or without antibiotic exposure, as well as in fish. The baseline gut microbiota of both snails and fish were established under a standardized set of experimental conditions, including a defined diet and a consistent water matrix. Since these conditions were consistent across all microcosms, the significant shifts in bacterial community composition we observed could

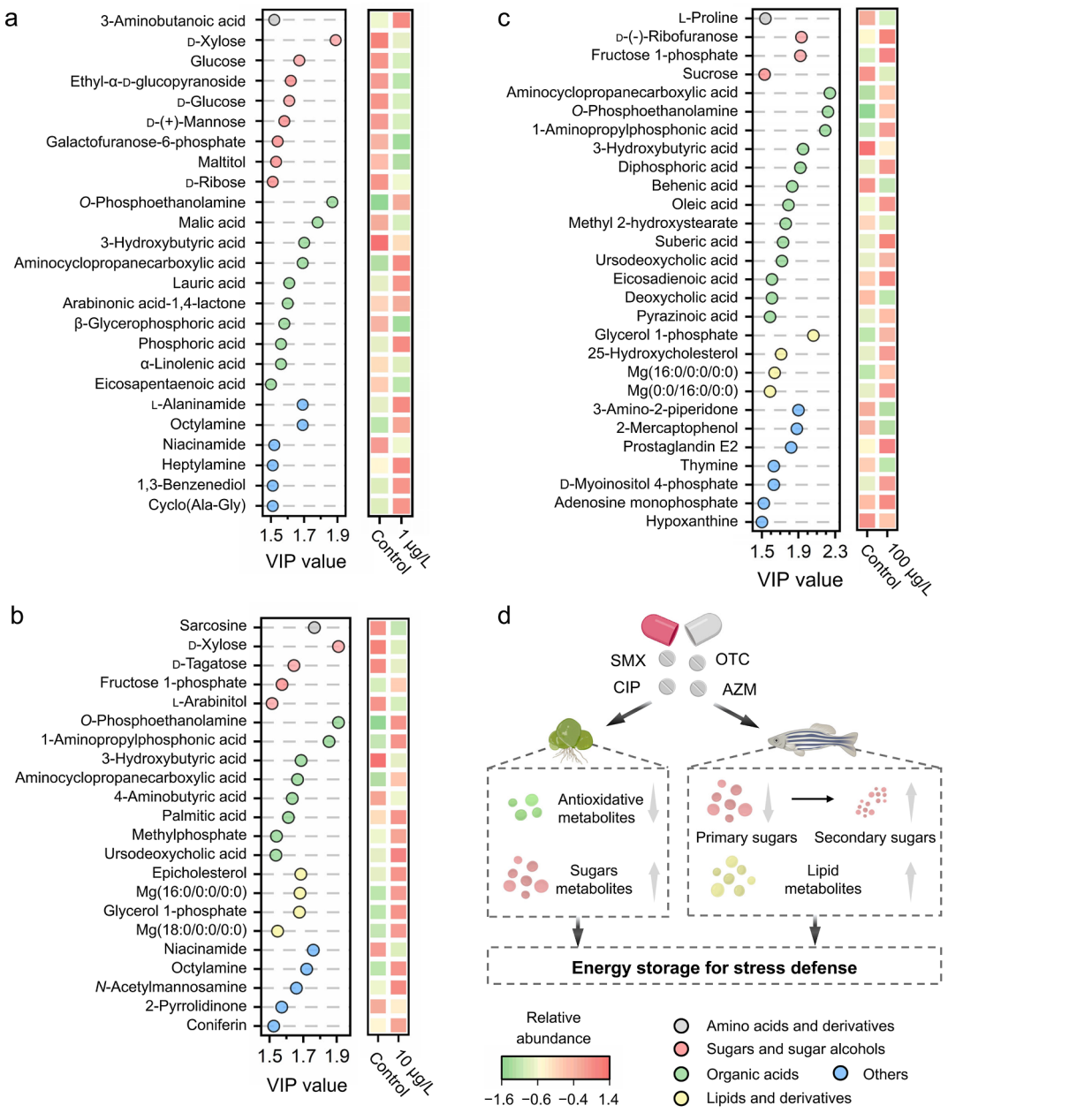


Fig. 3. The variable importance in projection (VIP) score and relative abundance of discriminated metabolites in the liver of zebrafish after exposure to mixed antibiotics each at (a) 1 μg/L, (b) 10 μg/L, and (c) 100 μg/L for 45 days. The livers of fish collected from the same microcosm were pooled as one sample ($n = 3$). And (d) a summary of metabolomic comparisons between duckweeds and fish liver under exposure to 100 μg/L of mixed antibiotics.

be reliably attributed to the antibiotic exposure. Upon antibiotic exposure, the richness and evenness of gut bacteria were not affected in either snails or fish (Figs. S10a and S11a), while the PCoA analysis and dissimilarity test indicated that the bacterial composition was significantly altered (Figs. S10b,c, and S11b,c). In these two aquatic organisms, the dominant bacteria at the phylum level (>1%) were the same, mainly including Proteobacteria, Fusobacteria, and Firmicutes (Fig. S12); however, the antibiotic-induced alteration of bacterial species was distinct. The abundance of Fusobacteria increased in snail gut after exposure to 1 and 10 μg/L of antibiotics, but decreased in the case of 100 μg/L. In comparison, Fusobacteria abundance showed antibiotic dose-dependent increase in fish gut. In terms of Firmicutes, its abundance posed an opposite tendency compared to Fusobacteria, both in snails and fish. Fusobacteria can provide vitamins or promote gut metabolism for the host [53], and Firmicutes can disrupt metabolic pathways involved in energy utilization and lipid homeostasis [54]. Therefore, we hypothesized that gut bacteria in snails might self-coordinate under exposure to 1 and 10 μg/L of antibiotics but dysregulate under exposure to 100 μg/L of antibiotics, while in fish, gut bacteria regulated positively in response to antibiotic exposure.

Gut bacteria at the OTU level further evidenced our hypothesis and aligned with the observed increase in triglyceride content in snails. In snails, the relative abundances of *Cetobacterium* (OTU_2, 3, 21, and 22) increased under exposure to 1 and 10 μg/L of antibiotics but decreased in the 100 μg/L case (Fig. 4a). *Cetobacterium* is capable of strengthening the host's immune system and promoting gut health through excretion of vitamins and antimicrobial metabolites [55,56]. However, several bacteria related to liver disease exhibited an increased abundance under 100 μg/L of antibiotic exposure (Fig. 4a). For example, the relative abundance of *Clostridium_sensu_stricto_1* (OTU_5), which is associated with intestinal inflammation [57], showed an opposite trend compared to *Cetobacterium*. The OTU_4 belonging to γ-proteobacteria became the

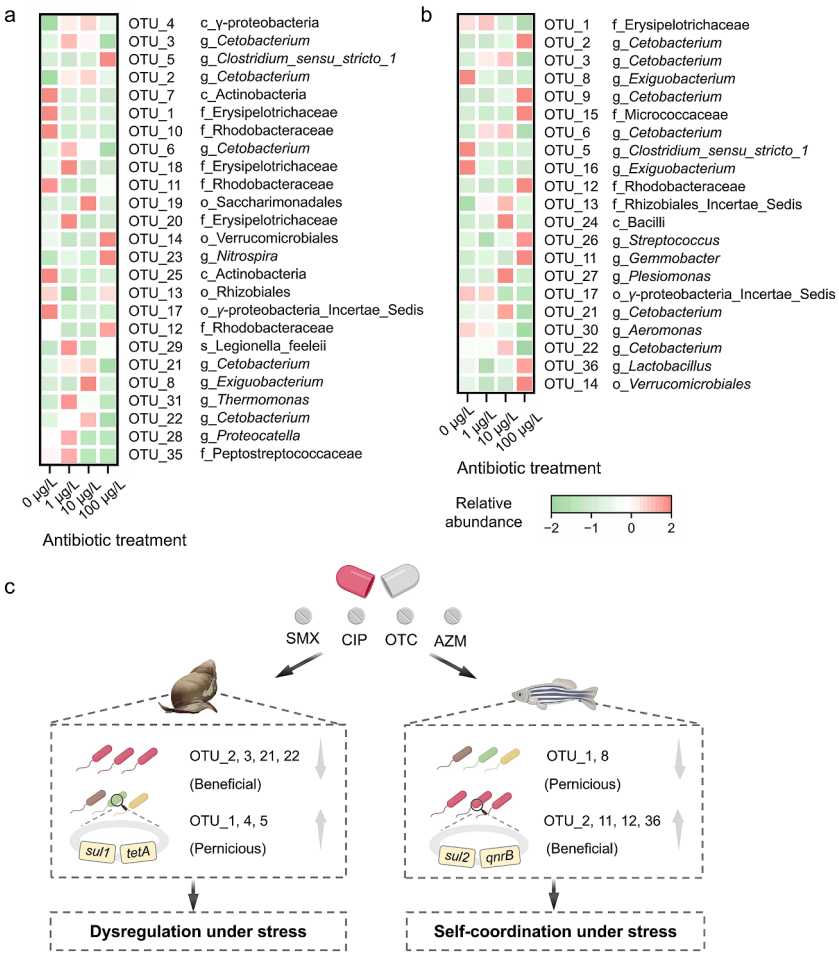


Fig. 4. The relative abundance of the dominant bacteria at the OTU level (>0.5%) in the gut of (a) snails and (b) fish across different groups after exposure to mixed antibiotics each at 0, 1, 10, and 100 μg/L for 45 days. The guts of snails or fish collected from the same microcosm were pooled as one sample (n = 3). And (c) a summary of the differences in gut bacterial responses between snails and fish under exposure to 100 μg/L of mixed antibiotics.

most abundant (>20%) after antibiotic exposure. The proliferation of proteobacteria in the gut was often associated with liver disease and obesity [58]. The shifts in dominant gut microbial taxa under 100 μg/L of antibiotic exposure further partly explain the observed triglyceride accumulation in snails (Fig. S6). Therefore, exposure to high levels of antibiotics might induce dysbiosis in the gut bacteria of snails, resulting in lipid accumulation and potential host disease.

In contrast, exposure to antibiotics at 100 μg/L increased the abundance of beneficial bacteria and decreased pernicious bacterial abundance within the fish gut, potentially improving the health of the host. On the one hand, OTU_2 belonging to *Cetobacterium* was significantly upregulated and became the most dominant genus (>50% of relative abundance) after exposure to 100 μg/L of antibiotics (Fig. S13). In previous studies, the effect of antibiotics on zebrafish gut bacteria in single-species systems demonstrated inconsistent changes in *Cetobacterium* abundance in response to antibiotic exposure, such as a decrease after 87 days of exposure to 500 ng/L of tetracycline [22] and an increase after 42 days of exposure to 260 ng/L of SMX or 420 ng/L of OTC [59]. Our study demonstrated the self-regulation of gut probiotics in fish when living with other aquatic organisms in receiving water contaminated with mixed antibiotics. This is further evidenced by the significant increase in other probiotics, such as *Lactobacillus* (OTU_36), which can regulate lipid metabolism and reduce liver fat content [60], and *Gemmobacter* (OTU_11), as well as Rhodobacteraceae (OTU_12), which can promote enzyme activity and vitamin synthesis to improve host nutrition and health [61]. On the other hand, the dominant bacterium OTU_1 (>41% of the relative abundance in the control group, family Erysipelotrichaceae) and OTU_8 (genus *Exiguobacterium*) significantly decreased in abundance (Figs. 4b and S13). These two genera were reported to be associated with lipid absorption and body adiposity [62, 63]. Thus, these dominant lipid-metabolizing gut microbiota in fish explained the findings that triglyceride levels were maintained in fish, contrasting with snails, where increased triglycerides might correlate with enriched lipid-accumulating gut microbiota (Fig. S6).

Given that the regulation of bacterial abundance may be partly attributed to their diverse resistance to antibiotics [19], we determined the abundance of eight ARGs and one MGE in the snail and fish gut bacteria. The results showed that the relative abundance of ARGs and MGE increased with increasing antibiotic concentration, especially *sul1* and *intl1*, which increased significantly after exposure to 100 μg/L of antibiotics (Figs. S14 and S15). Additionally, we constructed networks to correlate ARGs with the dominant gut bacteria in snails and fish, demonstrating that OTU_5, 11, and 14 in the snail gut (Fig. S16a) and OTU_2, 12, and 15 in the fish gut (Fig. S16b) might be the potential hosts of ARGs. Some bacteria (e.g., OTU_1 in the fish gut) exhibited a negative correlation with all the ARGs investigated, suggesting their disadvantageous niche under antibiotic stress. All these findings support the view that some gut bacteria were selectively enriched due to their antibiotic resistance, thereby influencing host health in either a positive or negative manner.

Taken together, the gut bacteria of snails and fish displayed a similar pattern of resistance to 1 and 10 μg/L of antibiotics but a distinct pattern to 100 μg/L of antibiotics. Under higher antibiotic exposure, the self-coordination of bacteria in the snail gut was disrupted, as evidenced

by a decrease in the abundance of beneficial bacteria and an increase in the abundance of pernicious bacteria (Fig. 4c). In contrast, the fish gut bacterial community was altered to mitigate antibiotic-induced effects. Several beneficial gut bacteria were involved in host health protection, such as OTU_2, which increased in the fish gut but decreased in the snail gut (Fig. 4c). The different enrichment pattern of gut bacteria in the two animals might be related to their distinct resistance to antibiotics. Gut bacterial communities must maintain a dynamic equilibrium to ensure the functioning of gut homeostasis and defense against pathology [19]. The observed diverse responses of gut bacteria in different organisms to antibiotic exposure highlight the need to evaluate gut microbiota dynamics in multilevel organisms when assessing the effects of antibiotic exposure on aquatic ecosystems.

3.6. Correlation between liver metabolites and gut bacteria in fish

Owing to the gut-liver axis, gut bacteria are involved in bile acid transformation and are closely related to lipid metabolism in the liver [34]. To better understand the interaction mechanism between liver metabolism and gut bacteria in response to antibiotic exposure, we took zebrafish as the representative and established a correlation network between the liver metabolites and the dominant gut bacteria (Fig. 5a). The beneficial bacteria OTU_2 and OTU_9, both belonging to *Cetobacterium*, were positively correlated with ursodeoxycholic acid, while the pernicious bacteria OTU_1 was positively correlated with deoxycholic acid. Similarly, ursodeoxycholic acid, a secondary bile acid known to modulate lipid accumulation in the liver [64], was also found to be positively related to the abundance of *Cetobacterium* in the gut of grass carp (*C. idella*) [65]. Deoxycholic acid, another secondary bile acid produced by gut bacteria, is known to cause DNA damage and may promote obesity-related diseases at high levels [66]. Therefore, these findings suggest a pivotal role of the gut-liver axis in mediating liver lipid accumulation induced by antibiotic exposure.

We further proposed a model for the potential self-regulatory mechanism in fish, through which the gut bacteria and liver metabolites might interplay via the gut-liver axis (Fig. 5b). Upon antibiotic exposure, lipid accumulation occurred in the liver as a result of glycogen decomposition. Simultaneously, primary bile acid precursors synthesized from cholesterol (e.g., 25-hydroxycholesterol) in the liver were transported to the gut, where they were transformed into secondary bile acids (e.g., ursodeoxycholic acid) by gut bacteria (e.g., OTU_2). These secondary bile acids were subsequently reabsorbed by liver cells through the gut-liver axis, which might subsequently participate in the positive regulation of excessive lipid accumulation in the liver. Thus, fish were proposed to exhibit a dynamic balance in response to antibiotic-induced stress: lipids were stored in the liver to defend against stress, while gut bacteria mitigated lipid accumulation to safeguard host health, potentially through production of secondary bile acids.

In previous studies, the gut-liver axis has been associated with pollutant-induced stress, but in a negative relationship. For example, a 14-day exposure to 10 mg/kg of perfluorooctane sulfonate regulated amino acid metabolism in mice liver via modulation of fecal microbiota through the gut-liver axis, further exacerbating liver disease [34]. Similarly, the gut-liver axis was identified as a potential target of microplastics (690 μg/L) and OTC (3 μg/L) in zebrafish, as evidenced by disruptions in liver metabolism and gut bacterial community [51]. In contrast, using an integrated approach combining metabolomics and microbiome analyses, our study provides evidence to support a critical and beneficial role of the gut-liver axis in fish in response to antibiotic defense. Future studies employing germ-free models or specific bile acid interventions are warranted to experimentally validate the causal relationships underlying the proposed gut-liver axis.

4. Conclusions

This study demonstrated the uniform and specific responses of multilevel aquatic organisms under exposure to antibiotics. For direct response, metabolites in duckweeds and fish liver were uniformly reprogrammed to store energy as a defense mechanism against antibiotic stress, while the reprogramming pathways were distinct. Antibiotics induced dysregulation of defense-related metabolites, along with the accumulation of total soluble sugars and various sugar-related metabolites in duckweeds, while primary sugar metabolites were decomposed in fish liver to facilitate lipid accumulation for energy storage. For indirect responses, the abundance of beneficial bacteria in the guts of both snails and fish increased under exposure to 1 and 10 μg/L of antibiotics, suggesting the self-coordination mechanism against antibiotic stress. However, the gut bacteria of snails were dysregulated under exposure to 100 μg/L of antibiotics, as evidenced by a decrease in beneficial bacteria and an increase in the pernicious bacteria, which resulted in triglyceride accumulation. On the contrary, the abundance of probiotics (such as *Cetobacterium*) in the fish gut showed a dose-dependent increase. This shift was associated with changes in bile acid metabolism that correlated with mitigated lipid accumulation in both the liver and whole body, a phenomenon possibly linked to microbial resistance to antibiotics. These findings suggest a potential beneficial role of the gut-liver axis in protecting aquatic organisms against antibiotic exposure.

To our knowledge, this is the first study to characterize uniform and specific dysregulation and adaptive defense behavior of multilevel organisms under antibiotic exposure, along with the underlying molecular mechanisms. Our integrated metabolomic and microbiome analyses suggested the significance of incorporating sublethal molecular responses across multilevel organisms to broaden the current effect assessment framework and future water quality criteria for antibiotics. While our study focused on individual responses in the freshwater

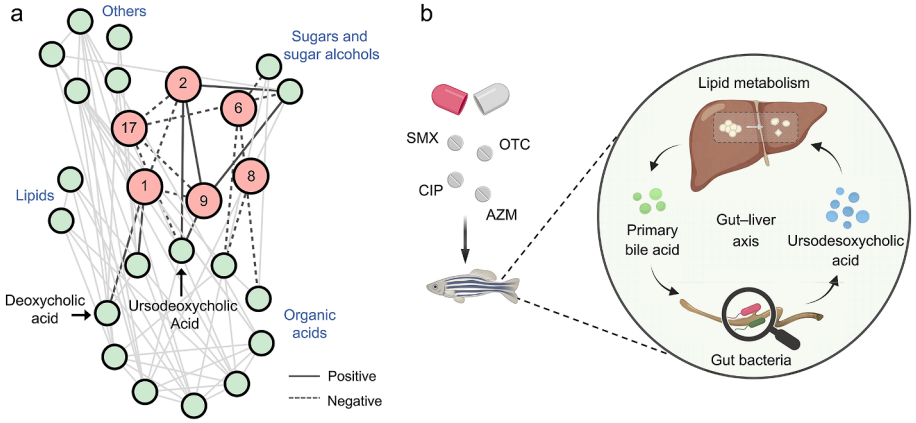


Fig. 5. The relationship between liver metabolomic response and gut bacteria change in fish after exposure to mixed antibiotics each at 100 μg/L for 45 days. (a) Network analysis of the relationship between liver metabolites (VIP >1.5 and $p < 0.05$; green circles) and dominant gut bacteria (relative abundance of >0.5%; red circles). The numbers in red circles represent the OTU number. Changed metabolites belonging to different categories are clustered. The significant positive and negative correlations ($p < 0.01$ and $r > 0.9$) are visualized with black solid and dashed lines, respectively. The correlation ($p < 0.01$ and $r > 0.9$) between metabolites is visualized with gray solid lines. (b) Schematic diagram of a potential mechanism by which gut bacteria-mediated bile acid metabolism and lipid metabolism interplay through the gut-liver axis in fish.

microcosm, the complex interspecies interactions and ecosystem-level effects remain unexplored. Future research should investigate these interactions within a more integrated ecosystem framework, incorporating more organism categories. Such studies would benefit from leveraging artificial intelligence and other advanced analytical techniques to better understand community- and ecosystem-level responses to antibiotic residues. Furthermore, expanding microbiome analysis to the phytomicrobiome of aquatic plants would improve our understanding of antibiotic impacts across all biological compartments in the ecosystem. Given the complexity of mixed exposure and the potential interactions between antibiotics, it is also essential to assess the contribution of each antibiotic to the overall phenotypic and molecular responses of aquatic organisms. Overall, our study provides novel insights into the uniform and specific molecular responses of aquatic organisms to antibiotic exposure, offering a suite of sensitive endpoints from the perspectives of metabolic reprogramming and microbial dysbiosis to support water quality benchmark development for antibiotic pollution control.

CRediT authorship contribution statement

Bin Wang: Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Hailing Zhou:** Validation, Methodology, Investigation, Formal analysis. **Jiayi Yang:** Validation, Methodology, Investigation. **Yushi Fang:** Validation, Methodology, Investigation. **You Zi:** Validation, Methodology, Investigation. **Lidan Zhang:** Methodology, Investigation. **Jiacheng Wang:** Investigation. **Lianhong Wang:** Methodology. **Yujie He:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Rong Ji:** Writing – review & editing, Supervision, Funding acquisition. **Tao Lyu:** Writing – review & editing, Methodology, Funding acquisition.

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.

Acknowledgments

Financial supports from National Key Research and Development Program of China (2021YFA0910300), the National Natural Science Foundation of China (22176090, 223B2602, 22436002, and 21806075), the Science and Technology Innovation Program of Jiangsu Province (BK20220036), State Key Laboratory of Pollution Control and Resource Reuse Foundation (PCRRF 22042), Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX24_0172), and the Program for Outstanding PhD Candidates of Nanjing University (2025A05) are kindly acknowledged. We thank Prof. Fenghua Wang for providing the plasmid for the qPCR experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2026.100231.

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2026-06
Elsevier
https://doi.org/10.1016/j.eehl.2026.100231
Wang B, Zhou H, Yang J, et al., (2026) Metabolomic and microbial responses of multilevel aquatic organisms to antibiotics in freshwater microcosm: the uniformity and specificity. Eco-Environment & Health, Volume 5, Issue 2, June 2026, Article number 100231

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