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Research article

Microbial community structure and antibiotic resistance profiles in sediments with long-term aquaculture history

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ABSTRACT

The aim of this investigation was to examine the microbial populations and their resistance patterns towards antibiotics, including the impact of nitrogen metabolism in response to the reintroduction of antibiotics, as well as the presence of resistance genes in sediments from shrimp ponds that have been utilized for extended periods of 5, 15, and over 30 years. Results showed that the sediments exhibited a high prevalence of *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi*, and *Oxyphotobacteria* as the most abundant bacterial phyla, accounting for 70.35–77.43% of the total bacterial community. The five most abundant phyla of fungi detected in all sediments, namely *Rozellomycota*, *Ascomycota*, *Aphelidiomycota*, *Basidiomycota*, and *Mortierellomycota*, constituted 24.26–32.54% of the total fungal community. It was highly probable that the *Proteobacteria* and *Bacteroidetes* phyla serve as the primary reservoir of antibiotic-resistant bacteria (ARB) in the sediment, which included various genera like *Sulfurovum*, *Woeseia*, *Sulfurimonas*, *Desulfosarcina*, and *Robiginitalea*. Among these genera, *Sulfurovum* appeared to be the most widespread in the sediment of aquaculture ponds that have been in operation for more than three decades, while *Woeseia* dominated in ponds that have been recently reclaimed and have a 15-year aquaculture history. Antibiotic resistance genes (ARGs) were categorized into seven distinct groups according to their mechanism of action. The prevalence of multidrug-resistant ARGs was found to be the highest among all types, with an abundance ranging from 8.74×10^{-2} to 1.90×10^{-1} copies per 16S rRNA gene copies. The results of a comparative analysis of sediment samples with varying aquaculture histories indicated that the total relative abundance of ARGs was significantly diminished in sediment with a 15-year aquaculture history as opposed to sediment with either a 5-year or 30-year aquaculture history. Another assessment of antibiotic resistances in aquaculture sediments involved an examination of the effects of reintroducing antibiotics on nitrogen metabolism processes. The findings revealed that the rates of ammonification, nitrification, and denitrification in the sediment with a history of 5 years and 15 years, decreased as the concentration of oxytetracycline increased from 1 to 300, and 2000 mg/kg, and inhibitory effects were found to be less pronounced in sediments with a 5-year history compared to those with a 15-year history. In contrast, oxytetracycline exposure led to a significant decrease in the rates of these processes in aquaculture pond sediments with a >30 years of aquaculture history across all the concentrations tested. The emergence and dissemination of antibiotic resistance profiles in aquaculture environments requires attention in future aquaculture management.

1. Introduction*

The extensive application of antibiotics in aquaculture has resulted in their ubiquitous existence in the surroundings, encompassing quinolones, sulfonamides, and tetracyclines, which poses a significant menace to aquatic ecosystems and human well-being (Xi et al., 2015; Klase et al., 2019; Wang et al., 2021; Hossain et al., 2022). Inappropriate application of antibiotics and the pollution of aquatic ecosystems exert a substantial influence on the configuration and constitution of the microbial community, culminating in a decline in the scope of taxonomic diversity or an alteration in the relative prevalence of diverse microbial groups (Martínez, 2017; Feng et al., 2022b; Wang et al., 2022). The effects of antibiotics on microbial groups may include a single species, such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Edwardsiella tarda*, or

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large communities of fungi or bacteria, resulting in changes in the relative abundance of microbial species and the interactions between different species (D'Costa et al., 2011; Czekalski et al., 2012; Xi et al., 2015; Bondarczuk and Piotrowska-Seget, 2019). Antibiotics also can reduce microbial population diversity by promoting the growth of antibiotic-resistant or -tolerant microbial strains under strong selective pressure (Kraemer et al., 2019). Lower antibiotic concentrations can promote the growth of bacterial strains with increased phenotypic and genotypic diversity, whereas intermediate concentrations of antibiotics may be associated with increased adaptability and resistance profiles in several bacterial species (Kraemer et al., 2019; Larsson and Flach, 2022).

The presence of antibiotic-resistant bacteria (ARB) with antibiotic-resistance genes (ARGs) is an added potential ecological hazard posed by antibiotics (Bhullar et al., 2012; Zhuang et al., 2021). It has come to attention that there has been a noticeable rise in the occurrence of antimicrobial resistance genes (ARGs) in environmental samples over the past few years. For example, soil samples collected from the Netherlands have exhibited a substantial increase in the prevalence of ARGs since the 1940s (Knapp et al., 2010). Antibiotic resistance can occur through a variety of mechanisms, such as inactivation of the antibiotic, efflux transport out of the bacterial cell, or modification of the antibiotic target (Zhuang et al., 2021; Larsson and Flach, 2022). The occurrence of de novo resistance evolution, wherein microorganisms acquire resistance to antibiotics as a result of human-induced selective pressures, has garnered significant interest in recent times (Larsson and Flach, 2022). Specifically, the widespread presence of antibiotic-resistant bacteria (ARB) and resistance mechanisms in the environment is believed to be primarily attributed to the extensive usage of antibiotics by humans (Kim and Cha, 2021; Pulingam et al., 2022). The evidence of antibiotic pollution and its consequential contamination of ecosystems with ARGs has been witnessed globally, spanning across various environments such as freshwater systems, estuaries, agricultural soils, and even Arctic regions (Kraemer et al., 2019; Larsson and Flach, 2022).

Aquaculture environments that have been continually exposed to high levels of antibiotics for extended periods are suspected to have increased levels of ARGs. Various studies have been conducted on the presence and propagation of ARGs in marine aquaculture ecosystems, with resistance to quinolone, tetracycline, and sulfonamide being found across these ecosystems (Gao et al., 2012; Jang et al., 2018; Zhuang et al., 2021). The elevated pressure of antibiotics has the potential to alter the growth of microorganisms, thereby facilitating the spread of antibiotic resistance genes (ARGs) through vertical gene transfer. Consequently, there exists a correlation between the composition of microbial communities and the dissemination of ARGs. Microorganisms are considered the primary carriers and determinant factors for the distribution and dissemination of ARGs, and key ARG carriers include the bacterial phyla *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Verrucomicrobia* (Xiong et al., 2015; Fang et al., 2019; Wang et al., 2022). However, aquaculture systems that employ different husbandry practices are likely to harbor distinct resistomes owing to variations in antibiotic usage, feed, pond management and environmental conditions (Xu et al., 2020; Wang et al., 2022). Additionally, recent research has found that antibiotic and metal resistance genes commonly co-occur in the genomes of bacteria in various sediment samples; furthermore, other environmental factors, such as temperature, nitrogen content, and organic matter, present in aquaculture sediments can also affect the dissemination of resistance (Singer et al., 2016; Li et al., 2021; Ekeleme et al., 2021; Xue et al., 2022). Therefore, further investigation is required to gain a comprehensive understanding of the resistance profiles and their relationship with these various factors in aquaculture environment.

In recent times, there has been growing interest in aquatic ecosystems, which has demonstrated that the development of antibiotic resistance has a significant impact on the operational dynamics of aquatic ecosystems, such as nutrient cycling, organic matter production, and pollutant degradation (Roose-Amsaleg and Laverman, 2016). A stable expression of ARGs may ensue in aquaculture fields as a result of the selective pressure generated by the continuous use of antibiotics. The presence of these resistance genes may confer upon the ecosystem the ability to endure even in the absence of antibiotics or under conditions of heightened antibiotic concentrations (Hou et al., 2015; Feng et al., 2022a). In our previous endeavors, we conducted controlled experiments to evaluate the ecological response of enzyme activities and microbial community to the reintroduction of antibiotics in aquaculture ponds. The results showed that sediments from newly reclaimed aquaculture ponds (approximately five years of aquaculture) were in the malleable stage of antibiotic resistance, those with 15 years of history were in the sub-stable stage of antibiotic resistance, and those with long-term history were in the stable stage of antibiotic adaptation or resistance (Feng et al., 2022a, 2022b). However, there is a scarcity of research that has centered on the attributes of antibiotic resistance profiles in natural settings, specifically regarding the reaction of nitrogen metabolism to antibiotic exposure.

Nitrogen metabolism plays a pivotal role in the maintenance of ecosystem functions and the provision of services related to primary production and water quality. The evaluation of ecosystem function and the effects of environmental pollution frequently involve the measurement of nitrogen metabolism and identification of the microorganisms responsible for these processes (Xiao et al., 2021; Li et al., 2023). Various approaches have been formulated to measure the possible rates of the nitrogen metabolic pathways of ammonification, nitrification, and denitrification in soil or sediment (Jaramillo et al., 2018). These methods usually involves the introduction of substrate-induced stimuli (e.g., urea, amino acid, or NO_3^- addition), followed by the measurement of products such as NH_4^+ for ammonification potential, NO_3^- or $NO_3^- + NO_2^-$ for nitrification potential, and N_2O or $N_2O + N_2$ for denitrification potential. The underestimation of activities is a characteristic of these methods, which can be attributed to the fact that NH_4^+, NO_3^-, and NO_2^- serve as substrates for consecutive processes (Molstad et al., 2007). Additionally, these techniques offer a fragmented comprehension of individual nitrogen metabolic processes that are closely and consecutively interconnected. Although isotope additions and long-term microcosm/mesocosm incubations can offer a more comprehensive representation of the scope and magnitude of nitrogen metabolism, these approaches can be quite expensive and/or require a substantial amount of time (Granger and Wankel, 2016). An approach centered on consecutive-N-processes, which utilizes microcosm measurements during short-term incubation to provide an integrated assessment of ammonification, nitrification, and denitrification, would be highly advantageous in the assessment of anthropogenic impact on sedimentary environments.

Henceforth, this study has identified three distinct types of aquaculture settings that vary in the length of aquaculture activity, for the purpose of examining alterations in the microbial community and their antibiotic resistance patterns. Our objective was to provide an inclusive and unified understanding of the resistance characteristics, encompassing genes (ARGs), microbial community and ecosystem functions (nitrogen metabolism), as well as their correlation with diverse environmental factors in the natural aquaculture environment. The occurrence of ARGs and the response of nitrogen metabolism, such as denitrification, nitrification and ammonification rates, were defined as the resistance profiles in this study. The relationship between antibiotic resistance and environmental factors was also investigated to evaluate the spread of antibiotic resistance in aquaculture.

2. Materials and methods

2.1. Materials and experimental design

Sediments were collected from shrimp aquaculture ponds in Zhangzhou city, Fujian province, China. The locations of these

aquaculture farms are shown in a previous study (Xi et al., 2015; Feng et al., 2022a). Sediments were divided into three categories based on their aquaculture history: Sediment A from shrimp ponds with a 5-year aquaculture history; Sediment B from shrimp ponds with an approximately 15-year aquaculture history; and Sediment C from shrimp ponds with >30 years of aquaculture history. The physicochemical properties of the three sediment types were measured according to the method of Feng et al. (2022a) and are presented in Table S1 (in Supplementary Material). Sediment samples (approximately 10–15 cm depth) were carefully retrieved from the aquatic environment using a Box Mud Harvester (Beijing New Landmark Soil Equipment Co., Ltd., China). Sediments from each ponds were obtained from five discrete subsamples of equal size. A total of 25 subsamples from five ponds with the same aquaculture history were combined, these samples were subsequently divided sufficiently into five pots as replicates. The collected sediment was maintained at 4 °C and immediately transported to the laboratory. The fresh sediment samples were passed through a 2.0 mm wet sieve to be used for further analysis.

2.2. Determination of microbial community

Each sample was collected from the same sediment by randomly sampling spoons 10 times, these 10 samples were combined well and labeled as one sample for further analysis. Immediately, DNA was extracted from each sediment sample using the E. Z.N.A.® Soil DNA Extraction Kit (Omega Bio-tek, Norcross, GA, US), according to the manufacturer's instructions. The DNA quality was detected using 2% agarose gel electrophoresis and stored at −20 °C.

The bacterial V4 region of the 16S ribosomal RNA genes was amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′); the ITS1 region was amplified using primers ITS5-1737F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2-2043R (5′-GCTGCGTTCTTCATCGAT-GC-3′). PCR was performed at an initial denaturation temperature of 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; following these cycles, a final extension step was conducted at 72 °C for 5 min. PCR reactions were performed in a total volume of 20 μL containing 4 μL of 5 × FastPfu Buffer, 2 μL of dNTPs (2.5 mmol/L), 0.8 μL each primer (5 μmol/L), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

PCR products were purified using the Gene JET DNA Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, U.S.) according to the manufacturer's instructions. In accordance with the DNA library preparation procedure (Thermo Fisher Scientific, Massachusetts, U.S.), the Ion Plus Fragment Library Kit 48 rxns was employed to construct the DNA library. The library was then subjected to sequencing on the Ion S5™ XL platform (Thermo Fisher Scientific), resulting in the generation of 400 bp/600 bp single-end reads with an accuracy surpassing 99.99%. The quality of the reads was verified using Cutadapt (Martin, 2011; V1.9.1, http://cutadapt.readthedocs.io/en/stable/), and chimeric reads were removed using the UCHIME Algorithm (Edgar et al., 2011; Haas et al., 2011; http://www.drive5.com/usearch/manual/uchime_algo.html). Operational taxonomic units (OTUs) were obtained by clustering with a 97% similarity cutoff using Uparse software (Edgar, 2013; v7.0.1001, http://drive5.com/uparse/). OTUs were subsequently matched with the SSU rRNA database (http://www.arb-silva.de/) to analyze bacterial community and Unitdatabase (https://unite.ut.ee/) to analyze fungi community according to the method of Mothur and SILVA (http://www.arb-silva.de/) and BLAST using Qiime software (Version 1.9.1; http://qiime.org/scripts/assign_ taxonomy.html), respectively (Altschul et al., 1990; Edgar, 2004; Wang et al., 2007; Kõljalg et al., 2013; Quast et al., 2013).

2.3. Determination of ARGs

The abundance of ARGs was detected using the SmartChip Real-time PCR System (WaferGen, U.S.), according to the method described by Zhu et al. (2013). In this study, a total of 296 primers (Table S3) were used for 16S rRNA genes, 285 ARGs, and 10 mobile genetic element (MGE) marker genes, including eight transposases and two integrons. Real-time PCR amplification was performed at an initial denaturation temperature of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; following these cycles, a final extension step was conducted at 72 °C for 5 min. Real-time PCR reactions were performed in a 100 nL volume containing Light Cycler 480 SYBR Green I Master Mix 1 × (Roche, U.S.), Nuclease-free PCR-Grade water, bovine serum albumin (4 μg/μL), DNA (5 ng/μL), and primers (1 μmol/L).

2.4. Determination of denitrification rate, nitrification rate, and ammonification rate

Each homogenized fresh sediment sample (15.0 g) from the same ponds was placed into 60 mL brown glass bottles, which were sealed with a silicone rubber septum and aluminum cap. Each bottle was incubated in the dark at 25 °C for 12 h. Following this, sediments with different aquaculture histories (sediments A, B, and C) were exposed to three concentrations (1, 300, and 2000 mg/kg) of OTC. Next, 10 mL of urea solution was injected into the glass bottles as a treatment, and 10 mL of deionized water was injected into the control bottle. The sealed glass bottles were evacuated and flushed with helium thrice to remove N_2; the headspace air was then replaced with a mixture of 2:1 helium: oxygen at a pressure of 1 atm to simulate the corresponding environmental air-water-sediment continuum. The bottles were sealed with Vaseline and the gas in the sealed bottles was replaced three times using a vacuum cleaning system (Beijing Shuaien Technology Co., Ltd.). Finally, the treatment bottles were cultured in an incubator at 25 °C for five days in the dark.

At the end of the exposure period, N_2 and N_2O concentrations in the bottles were measured using a gas chromatography system (Agilent® 7890A, Agilent Technologies, Santa Clara, CA, USA) which was equipped with an automatic sampling system. After each gas was detected, 40 mL of a 2 mmol/L KCl solution was added to each bottle and shaken for 60 min. The supernatant was obtained after centrifugation at 300 rpm for 5 min. Finally, the supernatant was passed through a 0.2 mm syringe cellulose acetate filter, and concentrations of NH_4^+–N and $NO_2^- + NO_3$–N were determined using a flow injection analyzer (QC 8500, Lachat®, Loveland, CO, USA).

2.5. Sediment characterization

Sediments were characterized by their total carbon content (TC), total nitrogen content (TN), total sulfur content (TS), pH, electrical conductivity (EC), and sediment size distribution, according to previous studies (Xi et al., 2015; Feng et al., 2022a). TC, TN, and TS were measured using an Elementar Analysensysteme GmbH (Vario MAX, Germany). The EC and pH were measured in a sediment/deionized water slurry at a ratio of 1:2.5, using a pH-EC meter (Accumet Excel XL60, Fisher Scientific Inc., USA). The particle size distribution of the sediment was analyzed using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK).

2.6. Statistical analysis

The microbial diversity of the samples was analyzed and community complexity was calculated in the fungal and bacterial microcosms using Shannon and Chao estimators, respectively. The top microbial community at phylum or genus level (that contributed more than 0.1% to the total microbial community) were selected for further analysis. To analyze the differences in ARGs in the sediments (Sediments A, B, and C), the relative abundance of ARGs was calculated using Equation (1). The response of nitrogen metabolism to OTC exposure in the sediment was described in terms of denitrification, nitrification, and

ammonification rates. These rates were calculated using Equations (2)–(4):

$$\text{The relative abundance of ARGs} = \frac{\text{the copies of ARGs}}{\text{the copies of 16S RNA}} \quad (1)$$

$$\text{Denitrification rate}\,(\text{mg N}/(\text{kg}\cdot\text{DW}\cdot\text{h})) = \frac{(N_2+N_2O)_{urea} - (N_2+N_2O)_{control}}{T} \quad (2)$$

$$\text{Nitrification rate}(\text{mg N}/(\text{kg}\cdot\text{DW}\cdot\text{h})) = \frac{\left((N_2+N_2O)_{urea} - (N_2+N_2O)_{control}\right) + \left(\left(NO_3^- + NO_2^-\right)_{urea} - \left(NO_3^- + NO_2^-\right)_{control}\right)}{T} \quad (3)$$

$$\text{Ammonification rate}(\text{mg N}/(\text{kg}\cdot\text{DW}\cdot\text{h})) = \frac{\left((N_2+N_2O)_{urea} - (N_2+N_2O)_{control}\right) + \left(\left(NO_3^- + NO_2^-\right)_{urea} - \left(NO_3^- + NO_2^-\right)_{control}\right)}{T} + \frac{\left(\left(NH_4^+\right)_{urea} - \left(NH_4^+\right)_{control}\right)}{T} \quad (4)$$

where N_2+N_2O is the concentration of N_2 and N_2O in the bottle, NO_3^- + NO_2^- and NH_4^+ are the concentrations of NO_3^- and NH_4^+ in the sediments, T is the incubation time (h), and DW is the weight of the dried sediment.

One-way analysis of variance (ANOVA) was used to test for significant differences between the OTC treatment (1, 300, or 2000 mg/kg) and control (0 mg/kg) in each sediment type; a two-way ANOVA was employed to test for significant differences in sediments with different aquaculture histories (5-, 15-, 30-year aquaculture histories) and OTC concentrations (0, 1, 300, or 2000 mg/kg). Tukey Differences (HSD) was applied as a post-hoc test for means at the level of 0.05. Pearson correlation analysis was performed using Origin software (version 9.1, Northampton, MA01060, USA) to analyze different ARGs in the sediment in correlation with the physiochemical properties of each sediment and the different genera present. Network analysis was performed using Gephi (version 0.9.2, WebAtlas, France) to analyze the correlation between the relative abundance of the seven ARG subtypes and the top five microbial genera. Trend-surface Analysis was performed using Origin (version 9.1, Northampton, MA01060, USA) to analyze denitrification rate, nitrification rate, and ammonification rate in sediments with OTC exposure in with respect to their corresponding aquatic history.

3. Results

3.1. Microbial diversity in sediments from different aquaculture histories

From all sediment samples, 130,106 bacterial sequences were detected and assigned to 5020 OTUs, whereas 102,633 fungal sequences were detected and assigned to 1579 OTUs. The largest number of unique bacterial species was observed in the sediment with a 30-year aquaculture history (sediment C), followed by the sediment with a 15-year aquaculture history (sediment B), and a 5-year aquaculture history (sediment A); the largest number of unique fungal species was observed in sediment C, followed by A, and B (Fig. S1 in Supplementary Material). The complexity of the microbial community in all sediments was evaluated using alpha components, including species richness (Chao1 index) and diversity (Shannon index) (Table 1). The richness and diversity of bacteria were higher than those of fungi in the same sediment sample. However, no significant differences were observed in bacterial/fungal richness and diversity with different aquaculture histories. With an extended aquaculture history, the diversity of the dominant fungi was observed to increase, whereas the bacterial diversity remained unaltered. Furthermore, the ratio of fungi to bacteria also displayed an increase, indicating a shift in the microbial community composition. To evaluate the variances in microbial communities across different sample types at the OTU level, PCA and NMDS ordination were utilized for β-diversity investigation. The outcome of the PCA analysis revealed a significant disparity in the bacterial community composition between sediment A and sediment B and C. However, there was an overlap in the fungal community between sediment C and either sediment A or B (Fig. S2 a, b in Supplementary Material). The findings were substantiated by NMDS graphs (Fig. S2 c, d in Supplementary Material), which demonstrated a distinct divergence in the bacterial community composition between sediment A and B or C. Moreover, the sediment samples of the fungi community were seen to form clusters within the newly reclaimed aquaculture ponds (Sediment A).

3.2. Microbial community structure in sediments from different aquaculture histories

The most abundant bacterial phyla in the sediments were *Proteobacteria*, *Bacteroides*, *Unidentified Bacteria*, *Planctomycotes*, *Chloroflexi*, *Oxyphotobacter, Firmicutes*, *Spirochaetes*, *Latescibacter*, and *Gemmatimonadetes*, which collectively constituted 86.12%–88.00% of the bacterial community (Fig. 1). In sediment A, *Proteobacteria* was the most prominent bacterial species, whereas sediment B displayed a high degree of relative abundance of *Chloroflexi*. In sediment C, *Latescibacillus* was the dominant species (Fig. 1). The practice of aquaculture had resulted in a notable shift in the composition of bacterial communities. Specifically, the relative abundance of *Latescibateri* and *Unidentified Bacteria* had steadily increased, while other phyla, such as *Proteobacteria* and *Bacteroidetes*, had exhibited a gradual decline in their relative abundance

Table 1
Fungal and bacterial community for sediment with different aquaculture histories.

Type	Fungi		Bacteria		Fungi/Bacteria	
	Chao1	Shannon	Chao1	Shannon	Chao1	Shannon
Sediment A	1398.57 ± 193.92	5.72 ± 1.31	4398.59 ± 367.98	9.49 ± 0.23	0.32 ± 0.04	0.60 ± 0.13
Sediment B	1261.79 ± 184.68	5.68 ± 1.71	4876.50 ± 112.48	9.89 ± 0.13	0.26 ± 0.03	0.57 ± 0.17
Sediment C	1395.57 ± 62.57	6.46 ± 0.86	4574.42 ± 302.61	9.50 ± 0.37	0.31 ± 0.01	0.68 ± 0.11

A is sediment with 5 years aquaculture history; B is sediment with 15 years aquaculture history; C is sediment with 30 years aquaculture history. There were no significant differences ($p < 0.05$) in three types of sediment (sediment A, sediment B, sediment C) with Tukey's HSD test for means.

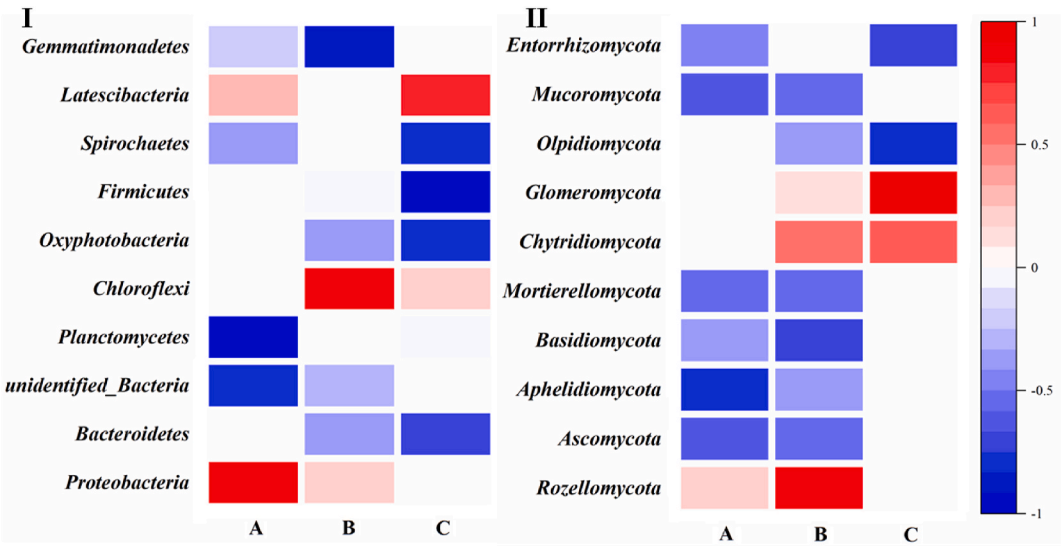

Fig. 1. Heatmap analysis of the relative abundance of bacterial community (I) and fungi community (II) in sediments with different aquaculture histories. A is sediment of shrimp ponds with a five years aquaculture history; B is sediment of shrimp ponds with a 15 years aquaculture history; C is sediment of shrimp ponds with a 30 years aquaculture history.

with the passage of time (Fig. S3 in Supplementary Material). The relative abundance of *Planctomycetes* was significantly higher in sediment B than in sediment A, whereas the relative abundance of *Oxyphotobacteria* was significantly lower in sediment B than in sediment A. A similar trend in the presence of *Chloroflexi* was observed to that of *Planctomycetes* (higher in sediment B than A). The most abundant genera in sediments, including *Sulfurovum*, *Woeseia*, *Sulfurimonas*, *Desulfosarcina*, and *Robiginitalea*, accounted for 10.88–21.96% of the total bacterial community. The relative abundance of these genera varied with aquaculture history (Fig. S3 in Supplementary Material), such that the relative abundance of *Sulfurovum* initially decreased and then sharply increased with prolonged aquaculture history, leading to a significant difference between sediments A, B, and C. The relative abundance of *Sulfurimonas* increased significantly in sediment C, but there was no significant difference between sediments A and B. Although other genera, such as *Woeseia*, *Desulfosarcina*, and *Robiginitalea*, exhibited slight variation, no significant difference was observed among sediments A, B and C.

The most abundant phyla of fungi across all sediments, including *Rozellomycota*, *Ascomycota*, *Aphelidiomycota*, *Basidiomycota*, *Mortierellomycota*, *Chytridiomycota*, *Glomeromycota*, *Olpidiomycota*, *Mucoromycota* and *Entorrhizomycota*, comprise 32.45–38.32% of the total fungal community (Fig. 1). Sediment A and B were observed to have a considerable abundance of the *Rozellomycota*, whereas Sediment C displayed a relatively high level of abundance for the *Glomeromycota*. With an increase in the duration of aquaculture, there was a gradual rise in the proportion of *Chytridiomycota* and *Glomeromycota* phyla, while the proportion of other phyla gradually declined. In comparison to sediments A and C, sediment B exhibited a significantly higher proportion of *Aphelidiomycota*, while other phyla, including *Mortierellomycota*, *Ascomycota*, and *Basidiomycota*, did not display any noteworthy differences among the three sediments (Fig. S3 in Supplementary Material). The most abundant genera of fungi, including *Tarzetta*, *Issatchenkia*, *Alternaria*, *Mortierella*, and *Aspergillus*, accounted for 11.70–13.01% of all fungal communities. The relative abundance of the five most common genera varied with aquaculture history (Fig. S3 in Supplementary Material), such that the relative abundance of *Issatchenkia* increased and subsequently decreased with a longer aquaculture history; a significant difference was observed between sediments A and B, and sediments B and C. The abundance of *Aspergillus* significantly decreased and later increased as aquaculture history progressed, whereas other genera showed no significant changes in sediments.

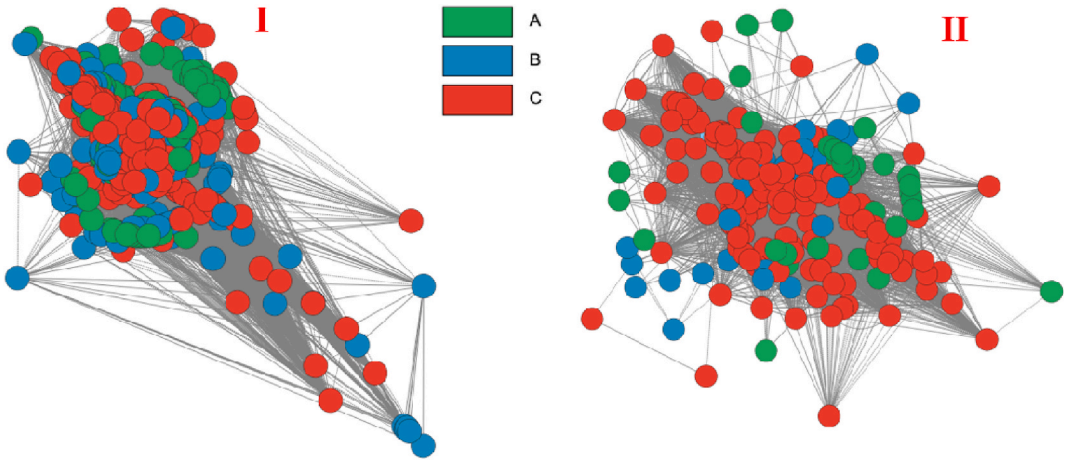

Fig. 2. Network analysis representing the microbial composition (I: bacterial; II: fungi) of sediment samples. A is sediment of shrimp ponds with a five years aquaculture history; B is sediment of shrimp ponds with a 15 years aquaculture history; C is sediment of shrimp ponds with a 30 years aquaculture history.

The results of the network analysis carried out at the genera level revealed a notable co-association of the microbial community, encompassing both bacteria and fungi, in the sediment C that was derived from long-standing aquaculture practices (more than 30 years) (Fig. 2). The sediment bacterial community in reclaimed aquaculture ponds (sediment A) seemed to be more concentrated and regulated, whereas the bacterial community in ponds that have been in operation for 15 and 30 years (sediment B and C) tends to develop in a specific direction. Nevertheless, there is no marked dissimilarity in the coexistence of fungal communities in the sediment of all three types of ponds (Fig. 2).

3.3. Occurrence of ARGs in sediments from different aquaculture histories

A total of 285 unique ARGs and 10 MGEs were detected in these sediment samples. The four major resistance mechanisms detected were deactivation, efflux, protection, and transposase (Fig. S4 in Supplementary Material). The most prevalent mechanism of antibiotic resistance among the studied bacteria was deactivation of the antibiotic (39.3%), followed by efflux (29.2%), and protection (24.4%). In all sediment samples, the prevailing ARG categories were those that aimed at aminoglycosides (namely gentamicin, kanamycin, and amikacin), beta-lactamase, multidrug, macrolide-lincosamide-streptogramin B (MLSB), tetracycline, vancomycin, and sulfonamide resistance genes (Fig. 3). The relative abundance of multidrug resistance genes was the highest, with values ranging from 8.74×10^{-2} to 1.90×10^{-1} copies/16S rRNA gene copies in sediments B and A, respectively. This was followed by the beta-lactamase and MLSB resistance genes. The proportion of ARGs associated with multidrug resistance initially decreased, but later increased with longer aquaculture history (e.g., 5, 15, and >30 years). Similar trends were observed for ARGs targeting tetracycline, sulfonamide, aminoglycoside, beta-lactamase, MLSB, and vancomycin (Fig. 3).

Of the unique genes in each class of ARGs, the *mexE* gene was the most prevalent in the class of multidrug resistance genes (Fig. S4 in Supplementary Material). The *aacA-aphD* gene, encoding resistance to aminoglycoside compounds, was found to be 11 times higher in sediment A than that in sediment B, and three times higher in sediment A than that in sediment C. For the beta-lactamase class, *blaROB* had the highest relative abundance with values of 2.08×10^{-2} and 3.30×10^{-3} copies/16S rRNA gene copies in sediments A and B, respectively. However, the *cfxA* gene had the highest relative abundance with values of 4.12×10^{-3} copies/16S rRNA gene copies in sediment C. Among the tetracycline antibiotic genes, the *tetE* gene had the highest relative abundance, from 1.28×10^{-3} to 7.88×10^{-3} copies/16S rRNA gene copies in sediments B and A, respectively. Among the 32 genes that confer resistance to vancomycin, the *vanB-02* gene was the most abundant; however, its abundance decreased by 79.48% in sediment B and 57.85% in sediment A. The *dfrA12* gene was present in seven of the sulfonamide-resistant genes analyzed, with the highest relative abundance found in sediment A (7.71×10^{-3} copies/16S rRNA gene copies) and the lowest in sediment B (3.17×10^{-4} copies/16S rRNA gene copies).

Transposase, chloramphenicol exporter, and integron genes were observed in all samples (Fig. 3). When compared to the relative abundance of transposase genes in sediment A, these were found to be 77.83% and 63.47% lower in sediments B and C, respectively. The genes responsible for the chloramphenicol exporter were more prevalent in sediment A than in sediments B and C, whereas the genes associated with integrons were more abundant in sediments A, but relatively less common in sediment B and C.

3.4. Response of denitrification rate, ammonification rate, and nitrification rate to OTC exposure

In sediment A, it was observed that exposure to OTC resulted in an initial rise in denitrification rate at a low concentration of 1 mg/kg, followed by a subsequent decline with increasing exposure concentration (Fig. 4). However, no significant variation was detected in high

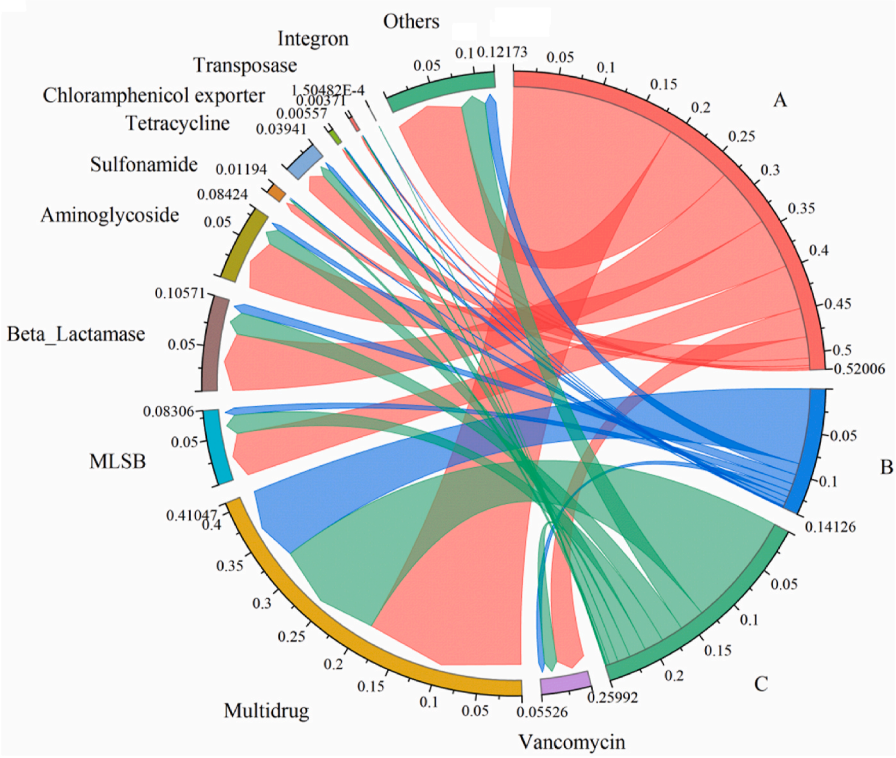

Fig. 3. Relative abundance of different antibiotic resistant geneses and mobile genetic elements in sediments of different aquaculture histories. A is sediment of shrimp ponds with a five years aquaculture history; B is sediment of shrimp ponds with a 15 years aquaculture history; C is sediment of shrimp ponds with a 30 years aquaculture history. MLSB means macrolide-lincosamide-streptogramin B.

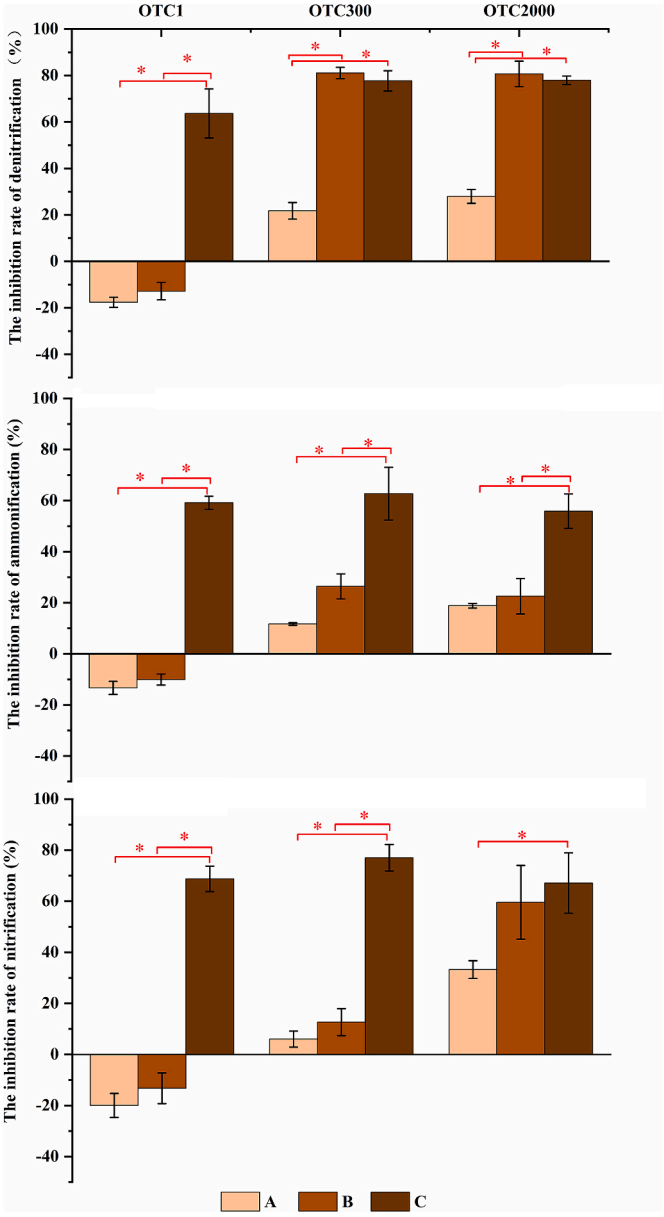

Fig. 4. The inhibition rate of nitrogen metabolism in sediments of different aquaculture histories with oxytetracycline exposure. A is sediment of shrimp ponds with a five years aquaculture history; B is sediment of shrimp ponds with a 15 years aquaculture history; C is sediment of shrimp ponds with a 30 years aquaculture history. * represents significant differences ($p < 0.05$) in sediment types (Sediment A, B and C). The inhibition rate = (the rate of treatment – the rate of control)/the rate of control*100%.

exposure concentrations ranging from 300 to 2000 mg/kg (Table S2 in Supplementary Material). In sediment B, the denitrification rate increased significantly at lower concentrations (1 mg/kg) of OTC exposure and subsequently decreased to less than half of the control level at higher exposure concentrations of 300 and 2000 mg/kg. In sediment C, the denitrification rate decreased significantly at all levels of OTC exposure. The effect of OTC exposure on denitrification rate exhibited heterogeneity across the three sediment types investigated. Sediments A and B demonstrated no discernible difference, while the response of denitrification rate to OTC exposure was significantly divergent between the exposure concentrations and the control. However, no statistically significant difference was detected at high exposure concentrations between 300 and 2000 mg/kg (Table S2 in Supplementary Material). In the control experiment, a comparable trend was observed in the ammonification and nitrification rates in response to OTC exposure, with distinct levels of inhibition were noted for each measured parameter. The rates of ammonification and nitrification increased significantly at the concentration exposure (1 mg/kg) in sediments A and B but decreased with higher exposure concentrations (300 and 2000 mg/kg) in both sediments. In contrast, OTC exposure led to a significant decrease in these rates in sediment C at all concentrations tested. The rates of ammonification and nitrification inhibition decreased as exposure levels increased in sediments A and B, whereas all OTC exposure levels seemed to be similar in sediment C (Fig. 4). Across the sediments from different aquaculture histories, the rates of ammonification in sediments A and B did not significantly differ when exposed to OTC, but there was a significant difference in sediment C. However, the nitrification rates among the three sediments were significantly different from each other (Table S2 in Supplementary Material).

4. Discussion

4.1. Shifts of microbial community in aquaculture environment

Aquaculture facilities are highly dependent on antibiotics to control infectious diseases which could otherwise jeopardize food production (Yang et al., 2019; Reverter et al., 2020; Shen et al., 2020). Consequently, aquaculture sediments are often reservoirs of antibiotics; moreover, the development of ARGs and ARB has become a major environmental contamination issue (D'Costa et al., 2011; Bhullar et al., 2012; Yuan et al., 2019; Chen et al., 2022; He et al., 2022). The continued use of antibiotics may alter the microbial community composition, potentially resulting in different antibiotic resistance profiles in the ecosystem (Feng et al., 2022b; Li et al., 2022). Aquaculture systems harbor a diverse range of microorganisms within their sediments, and the application of antibiotics in these systems can lead to a sustained selection pressure on the microbial community, ultimately resulting in a stable community structure as evidenced by this study (Martínez, 2017; Baquero and Levin, 2021; Feng et al., 2022b; Wang et al., 2022) (Table 1). The results showed that antibiotic residues can have bactericidal activity against specific bacteria, leading to the formation of antibacterial-resistant communities after long-term antibiotic use (Tao et al., 2016). These communities are typically composed of *Proteobacteria* and *Bacteroidetes*, which are known to harbor ARGs (Wexler, 2007; Liu et al., 2019). This study found that *Proteobacteria* and *Bacteroidetes* were the dominant bacterial phyla in all sediment samples (Fig. S3 in Supplementary Material), indicating that antibiotic selection in aquaculture environments leads to the formation of resistance profiles. *Proteobacteria* are highly susceptible to antibiotics and play a role in the development of antibiotic resistance in many research settings (Szekeres et al., 2017; Behnami et al., 2018; Elokil et al., 2019; Xing et al., 2022; Feng et al., 2022b); contrastingly, *Bacteroides* species are intrinsically resistant to various antibiotics (Wexler, 2007). The results further documented that *Woeseia* and *Robiginitalea* genera, which are classified into the *Proteobacteria* and *Bacteroidetes* phyla respectively, had a stronger positive correlation with six ARG subtypes (*aacA-aphD*, *blaROB*, *cfxA*, *dfrA12*, *tetE*, and *vanB-02*) than other genera (Fig. 5).

Antibiotics can inhibit the growth of closely related bacteria and spores and have shown fungicidal activity in some cases (Demoling et al., 2009; Yan et al., 2011; Cavera et al., 2015). The increased richness (Shannon index) of fungi with aquaculture history in natural settings indicated that the fungal community was less affected by antibiotic residues (Table 1). Fungi are increasingly being shown to be able to work in conjunction with extracellular and intracellular enzymes to transform the structures of many toxic xenobiotic compounds, including antibiotics, via various mechanisms (Olicón-Hernández et al., 2017; Gao et al., 2020; Ahumada-Rudolph et al., 2021; Wang et al., 2023). In this study, the five most abundant phyla of fungi that are *Rozellomycota*, *Ascomycota*, *Basidiomycota*, *Mortierellomycota* and *Aphelidiomycota*, were found to be resistant to antibiotic residues during aquaculture

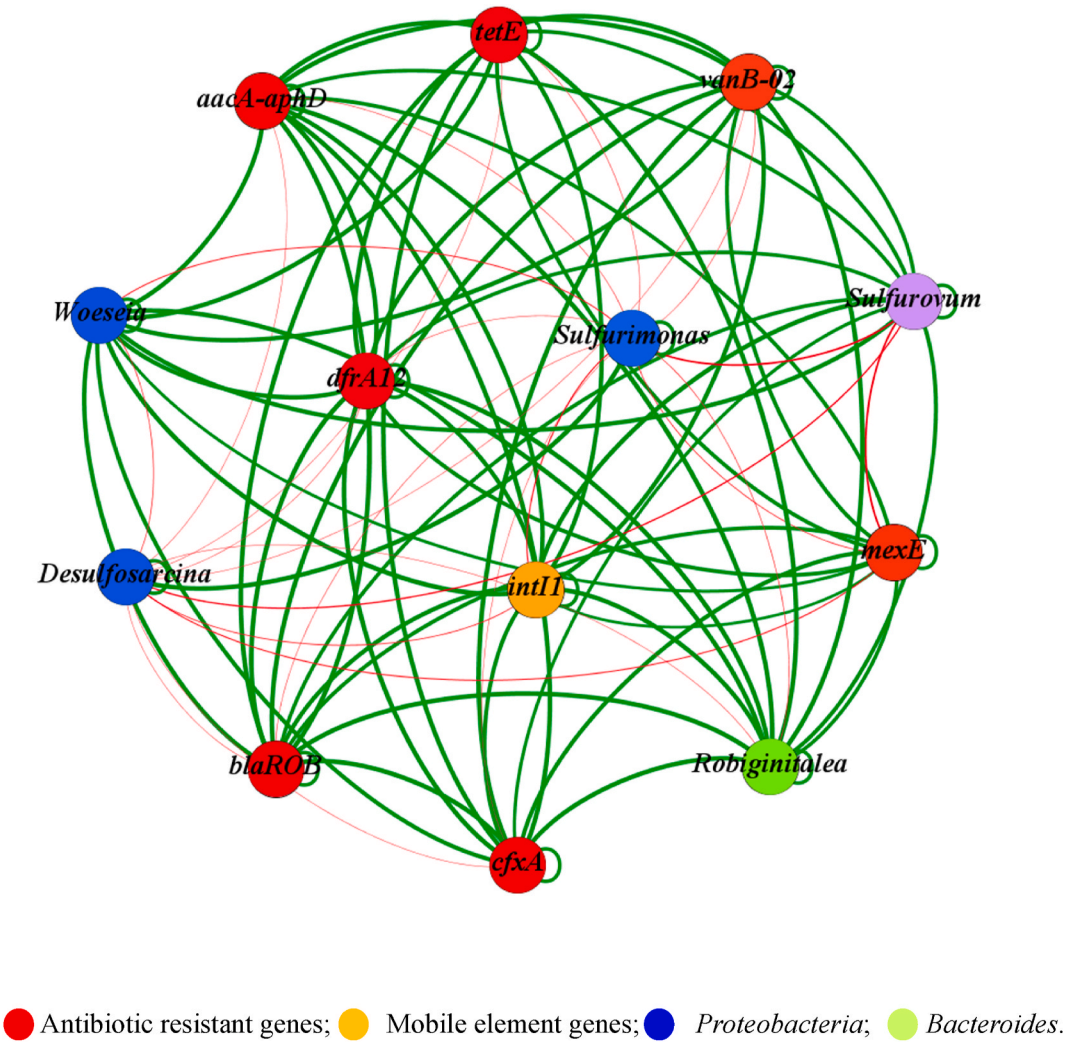

Fig. 5. Network analysis showing the relationships between antibiotic resistant genes, mobile element gene and bacterial microbial.
A connection was based on Pearson's correlation coefficient. Green line means a positive relation between each other; red line means a negative relation between each other.

management. Similar results showed that fungi belonging to different phyla (e.g., *Basidiomycota*, *Ascomycota* and *Mucoromycotina*) could remove and transform various pharmaceutical compounds at different rates. These fungi can produce hydroxylated, conjugated, and oxidized metabolites of pharmaceuticals, including antibiotics, psychiatric drugs, anticonvulsants, anti-inflammatory agents, and estrogens (Olicón--Hernández et al., 2017). Antibiotics can cause a significant decrease in the number of soil bacteria, leading to a dose-dependent increase in the fungal:bacterial ratio (Thiele-Bruhn and Beck, 2005; Hammesfahr et al., 2008). Similar findings were observed in the current study, whereby the fungal:bacterial ratio increased with prolonged aquaculture history. Similar resistance profiles of microbial community were also found in this aquaculture area in a natural setting, as well as in ecological responses to antibiotic re-entry experiments in previous studies (Xi et al., 2015; Feng et al., 2022a, 2022b).

4.2. Occurrence of ARGs in aquaculture environment

Aquaculture sediments are considered as reservoirs for various ARGs (Huerta et al., 2013; Cui et al., 2016; Niu et al., 2016; Qiu et al., 2021), and ARGs are enriched significantly under conditions of persistent antibiotic use compared to sediments that are scarcely affected by human activities (Szekeres et al., 2017; Tan et al., 2018; Xiong et al., 2018; Chen et al., 2019; Miao et al., 2022). Additionally, the ARG type was found to be associated with antibiotic resistance (Tao et al., 2016). The study revealed the existence of seven different types of ARGs, with multidrug resistance genes being the most prevalent and found in all sediments (up to 17.63%). The other primary resistance genes targeted MLSB (*erm*, *vat* genes), beta-lactamase (*blaROX*, *blaCTX-M* genes), aminoglycoside (*aac*, *aph* and *aad* genes), vancomycin (*van* genes), sulfanilamide (*dfa*, *sul* genes), and tetracycline (*tet* genes) (Fig. 3). Some ARGs belonging to MGEs have also been discovered and potentially promote the spread and integration of various ARGs into bacterial communities (Gillings, 2014; Ma et al., 2017; Li et al., 2018), resulting in the co-occurrence of different types of ARGs by strong genetic linkages (Jia et al., 2017). He et al. (2014) found that MGEs could contribute to the development of co-resistance or multidrug resistance.

The levels of ARGs varied among the sediments from different aquaculture histories, with the highest antibiotic resistance in sediment A, the lowest in sediment B, and that of sediment C was in between these two extremes, suggesting that antibiotic resistance was gradually induced and acquired in sediment A, reduced in sediment B, and maintained in sediment C (Fig. 3). An explanation for the development of antibiotic resistance is the overuse of antibiotics, which reduces the effectiveness of antibiotics against bacteria (Lu and Lu, 2020; Zhou et al., 2021). The decrease in the abundance of other ARGs in sediments

B and C was due to the lower abundance of the *intI1* gene, which is commonly located on transposons and plasmids and is closely related to multidrug resistance genes and other ARGs. This gene is a common indicator of antibiotic resistance (Zhang et al., 2016; Duan et al., 2017). The study revealed that, although the *intI1* gene had a lower relative abundance, it possessed a stronger positive relationship with seven ARG subtypes (*aacA-aphD*, *blaROB*, *cfxA*, *mexE*, *dfrA12*, *tetE*, and *vanB-02*) (Fig. 5). This suggests that the *intI1* gene plays a crucial role in the dissemination of ARGs in aquaculture sediments. A similar significant positive correlation between ARGs and the presence of the *intI1* gene has been observed in many natural settings (Chen et al., 2019; Qiu et al., 2021; Amarasiri et al., 2022). Moreover, the decreased relative abundance of other mobile element genes (*tnpA* and *IS613* genes) may have inhibited or reduced horizontal gene exchange via MGEs (Baquero et al., 2008; Marti et al., 2014; Gillings et al., 2015), further reducing the abundance of other ARGs in sediments B and C.

The different types of ARGs displayed three major resistance mechanisms: efflux pumps, antibiotic deactivation, and cellular protection (Fig. S4 in Supplementary Material). This is consistent with previous results for aquaculture environments (Bueno et al., 2019; Wang et al., 2019). Although the same types of ARGs may confer resistance to different antibiotics, they may do so via different mechanisms. This highlights the potential for cross-resistance development. For example, the *aacA-aphD* gene confers resistance to aminoglycosides by inactivating them, whereas the *blaOXA* gene confers resistance to beta-lactams by hydrolyzing them. Multidrug, MLSB, and tetracycline resistance are mediated by *acrA*, *mefA*, and *tetE*, respectively. The presence of different antibiotics in aquatic environments may exert selective pressure on bacterial populations, resulting in the formation of ARGs with multidrug resistance (Szekeres et al., 2017; Meng et al., 2019; Zayda et al., 2020; Gan et al., 2021). Multidrug resistance genes encode resistance mechanisms against more than one type of drug or antibiotic. This resistance is conferred via three mechanisms: efflux pumps, antibiotic deactivation, and cellular protection (Tanwar et al., 2014). Studies have observed similar resistance profiles in drinking water (Jia et al., 2015), paddy soils (Xiao et al., 2016), groundwater near landfills (Chen et al., 2017), natural water bodies (Liu et al., 2018), and cropland soils (Du et al., 2020).

Environmental factors such as pH and nutrient elements have been documented to directly affect the transfer of ARGs between cells through conjugation (Qiu et al., 2012; Di Cesare et al., 2015; Acosta-González and Marqués, 2016). The results showed that pH plays an important role in the variation of ARGs (Fig. S6 in Supplementary Material), which is consistent with previous studies that have confirmed that soil properties can directly affect the ARG composition by influencing the frequency of horizontal gene transfer among bacteria (Zheng et al., 2021). The relevance of nutrient elements, specifically organic matter, lies in their ability to influence the microbial community and promote the expression of ARGs (Thevenon et al., 2012; Li et al., 2020; Zhang et al., 2020). However, the study results showed that solely the total nitrogen content (TN) was linked to bacterial *Robiginitalea* (Fig. 6 in Supplementary Material), and the total carbon content was related to the mexE gene (Fig. S6 in Supplementary Material).

4.3. Response of nitrogen metabolism to oxytetracycline exposure

The presence of antibiotics in sediments can exert a long-term selection pressure on the microbial community, resulting in the formation of resistance or tolerance profiles. Consequently, the response of the microbial community to the reintroduction of antibiotics can vary greatly, resulting in significant changes in the biogeochemical functioning of ecosystems; these include processes such as nutrient cycling, organic matter production, and the degradation of pollutants (Roose-Amsaleg and Laverman, 2016; Li et al., 2022). In this study, the rates of ammonification, nitrification, and denitrification in sediments of newly reclaimed aquaculture ponds (approximately five years of aquaculture, sediment A) decreased with increasing concentrations of OTC; contrastingly, in sediment from aquaculture ponds with 15 years of history (sediment B), low concentrations of OTC promoted the nitrogen transformation process, but no changes were observed with OTC exposure at the higher concentrations. The nitrogen metabolism rates in sediments from aquaculture ponds with a long-term history (sediment C) were not significantly affected by exposure to OTC. These findings suggest that the ability of microbial communities to respond to OTC exposure stress diminishes with prolonged aquaculture history, and continued exposure to antibiotic selection pressure over the years appears to result in the increased antibiotic tolerance of these microbial communities. However, the microbial community can respond to antibiotic exposure differently in various ecosystems owing to large physicochemical differences that result in distinct responses to antibiotic exposure (Wang et al., 2020; Sun et al., 2017; Ma et al., 2016; De Vries and Zhang, 2016; Cui et al., 2016; Yang et al., 2012). Sedimentary ecosystems are complex, dynamic, and support a high diversity of microbes. The antibacterial molecules present in these ecosystems can be inactivated by interactions with organo-mineral compounds, organic matter, or reactions such as photodegradation or hydrolysis (Tang et al., 2019; Harrower et al., 2021; Maghsodian et al., 2022).

The three studied biogeochemical processes in nitrogen metabolism, including nitrification, anammox, and denitrification rates, represent distinct processes regarding metabolism and microbes. Denitrifying microbes are represented across three domains, whereas those that support nitrification or anammox have much less taxonomic diversity (Roose-Amsaleg and Laverman, 2016). Differences in microbial diversity might affect community susceptibility to antibiotic exposure, with multispecies communities potentially displaying greater resilience to antibiotics (Delgado-Baquerizo et al., 2016; Trifonova et al., 2021). Moreover, the rates of denitrification, ammonification, and nitrification in sediment A and B significantly decreased with an increase in OTC concentration (Fig. 4). The significant inhibitory effects of OTC on sediment-dwelling bacteria may be explained by a reduction in protein synthesis, enzyme activity, or gene abundance in these communities (Evaggelopoulou and Samanidou, 2013; Yin et al., 2017; Chen et al., 2015; Wang et al., 2020). Further investigation into the nitrogen metabolism of the targeted microbial community and their associated functional genes is imperative in order to elucidate the denitrification, ammonification, and nitrification processes in the context of antibiotic re-entry within an environment that already exhibits antibiotic resistance profiles.

5. Conclusion

The work presented an integrated comprehension of the antibiotic resistance features, which incorporates genes (ARGs), microbial community and ecosystem functions (nitrogen metabolism), and their association with various environmental factors in the natural settings. The impact of aquaculture history on the bacterial community was more profound than that on the fungal population, with *Sulfurovum*, *Woeseia*, *Sulfurimonas*, *Desulfosarcina*, and *Robiginitalea* being the most abundant bacterial genera, while *Tarzetta*, *Issatchenkia*, *Alternaria*, *Mortierella*, and *Aspergillus* comprising the primary fungal population. Sediments obtained from newly reclaimed aquaculture ponds exhibited the highest abundance of resistance genes, while those with a history of 15 years demonstrated the lowest levels. Sediments with a long-term history (more than 30 years) displayed intermediate levels of ARGs, falling between the two extremes. Nevertheless, the resistance of these sediment environments was found to be associated with the ecosystem response to these genes, including the *intI1* gene, as well as sediment properties, such as pH value. The observed antibiotic resistance patterns in diverse aquaculture environments were also linked to the suppressive effects of nitrogen metabolism, such as ammonification, nitrification, and denitrification, on antibiotic reintroduction. It was noted that these suppressive effects were less pronounced in sediments with a 5-year

history compared to those with a 15-year history, while a significant reduction in the rates of these processes was observed in aquaculture pond sediments with a history of >30 years across all concentrations tested. Ultimately, the development and spread of antibiotic resistance profiles in aquaculture environments is a crisis that must be addressed in future aquaculture management.

Credit author statement

Yongshan Chen: designed the work plan; Yue Lu and Jinghua Xu: performed all experiments; Ying Feng and Yongshan Chen: analyzed the data and wrote manuscript; Jinping Jiang: helped in modifying this manuscript. All authors read and approved the final manuscript.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.118052.

Acronym lists

Sediment A	sediments from shrimp ponds with 5 years of aquaculture history
Sediment B	sediments from shrimp ponds with a history of 15 years
Sediment C	sediments from shrimp ponds with a history of more than 30 years
ARB	antibiotic-resistant bacteria; ARGs:antibiotic-resistance genes
OTUs	Operational taxonomic units
TC	total carbon content
TN	total nitrogen content
TS	total sulfur content
EC	electrical conductivity
D	Vol. weighted mean value of particle diameter
OTC	oxytetracycline

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