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Effects of Three Antibiotics on Nitrogen-Cycling Bacteria in Sediment of Aquaculture Water

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Abstract: Antibiotics are commonly used to prevent and control aquaculture diseases. However, long-term overuse of antibiotics not only leaves residues but also leads to changes in the nitrogen cycle in water, which threatens the survival of aquaculture organisms. The current results showed that sulfamethoxazole had no significant effect on the nitrogen cycle process in the actual aquaculture concentration. The inhibitory effect of 1.05 mg/L norfloxacin on ammonia-oxidizing bacteria was significantly greater than that on ammonia-oxidizing archaea, and the gene abundance of *AOB amoA* on the 14th day increased by 2.48 times compared with the 7th day. Under the influence of 3.9 mg/L oxytetracycline, the gene abundance of *AOB amoA* decreased significantly, while the number of *AOA amoA* genes increased, suggesting that there may be functional redundancy between AOA and AOB. At the genus level in the norfloxacin group, the relative abundance of *Sva0485* increased by 14.0% on the 7th day compared with the control group but decreased 12.77% in the addition group. The relative abundance of *Firmicutes*, another dominant species in the oxytetracycline group, was 25.9%. This study shows that the addition of antibiotics may have a negative effect on the nitrogen-cycling microorganisms in aquaculture water.

Keywords: sulfamethoxazole; norfloxacin; oxytetracycline; aquaculture water; nitrogen cycle; gene abundance; community structure

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1. Introduction

Environmental pollution caused by antibiotics has been an important issue for several decades [1]. The misuse or overuse of antibiotics has led to the entry of a large number of antibiotics into water, and antibiotics have become one of the most critical emerging pollutants in aquatic environments [1]. Hundreds of antibiotics have been detected in various water bodies, such as surface water, seawater and groundwater, even drinking water [2]. The antibiotics released into wastewater may adsorb from suspended particles or sediments and eventually accumulate in the sediments of aquaculture water [3]. The residues of antibiotics may exert selective pressure on the bacterial community in the surrounding environment, accelerating the production of antibiotic-resistant bacteria (ARBs) and the transmission of antibiotic resistance genes (ARGs) [4], which pose a potential threat to health. Therefore, the potential risks posed by ARB and ARG pollution have also received extensive attention from scholars from all walks of life [5]. Harmful substances (such as

antibiotics) produced by human activities are important factors limiting the sustainability of aquaculture environments [6].

When antibiotics are present in the environment, they hinder the structure and function of microbial communities in different ways and have direct (short-term) and indirect (long-term) effects on microbial communities [7]. The influence of antibiotics on the structure and function of the microbial community in aquaculture water and interference with the microbial-driven nitrogen cycle have become important factors affecting eutrophication and greenhouse gas emissions [8]. The eutrophication of water bodies is mainly due to the imbalance of nitrogen and phosphorus contents in water bodies, and nitrogen is mainly removed by the metabolic activities of functional bacteria of nitrification and denitrification. The intensity of the metabolic activities of functional bacteria of nitrification and denitrification is related to their abundance in the ecosystem and the species composition of functional microorganisms of nitrification and denitrification in the environment [9]. Therefore, the abundance and community structure of nitrification and denitrification-related functional microorganisms in sediments affect the nitrogen content of the entire ecosystem. Therefore, the potential risks caused by antibiotic pollution have also received extensive attention from scholars from all walks of life.

At present, a large number of studies have reported the effects of antibiotics on microorganisms. Inhibitory effects on nitrification and denitrification rates have been observed in soils and sediments treated with commonly used antibiotics at therapeutic concentrations (such as norfloxacin (NOR), sulfadiazine (SDZ) and tetracycline (TC)) [10,11]. Antibiotics can reduce nitrification and denitrification rates through different mechanisms, including inhibiting microbial growth, destroying cell membranes, reducing electron transfer efficiency and reducing denitrification enzyme activity [12]. Sulfonamides are often used as antibiotics in aquaculture to prevent bacterial diseases, but their gradual accumulation has been shown to affect the microbial community and lead to the spread of ARGs [13]. By examining the effects of oxytetracycline stress on functional genes of denitrification, microbial communities and metabolic pathways in sediments, it was observed that oxytetracycline significantly inhibited the abundance of the *nirK* and *nosZ* genes and reduced the abundance of nitrate reductase, nitrite reductase and N_2O reductase [14]. Norfloxacin is a fluoroquinolone antibiotic, a category that includes ciprofloxacin and ofloxacin, which can cause irreversible damage to bacterial DNA [15,16]. Dai [17] reported that when the concentration of ciprofloxacin increased to 10 mg/L, the removal rate of ammonia nitrogen decreased by 19.11%. Li [18] found that when the concentration of norfloxacin was 30 mg/L, the contents of reactive oxygen species (ROS) and lactate dehydrogenase (LDH) in sludge increased by 22.3% and 22.5%, respectively. A higher concentration of ROS can disrupt the balance between oxidation and antioxidation in the system and subsequently cause toxicity to microorganisms. The increase in LDH indicates that the presence of norfloxacin leads to the rupture of the microbial cell membrane.

Antibiotic exposure may lead to changes in the relative abundance of microbial species and interactions between different species [19,20]. Feng et al. [21] found that OTC exposure had varying effects on microbial diversity in sediments by analyzing the responses of microbial gene abundance. In addition, antibiotics can influence the metabolic performance of microcosms. Tong et al. [22] reported that tetracycline and ofloxacin can affect the metabolic performance of microorganisms by inhibiting amino acid metabolism and carbohydrate metabolism. The stability of nitrogen cycling in the microcosms might be related to the changes in these functional performances. Many studies have shown that the effect of antibiotics on nitrogen-cycling microorganisms has the characteristics of 'low promotion and high inhibition', but low concentrations and high concentrations have different thresholds according to different studies. Cui [23] found that low concentrations (1 $mg \cdot kg^{-1}$) and high concentrations (>5 $mg \cdot kg^{-1}$) of ciprofloxacin increased and inhibited nitrification, respectively; however, a study showed that a mixture of tylosin and oxytetracycline promoted nitrification at low concentrations (50–100 $mg \cdot kg^{-1}$), while nitrification was inhibited at 150–700 $mg \cdot kg^{-1}$ [24]. The promotion of nitrification by low concentrations of antibiotics

may be derived from the redundancy of microbial populations. Archaea and fungi can surpass bacteria after bacteria are inhibited by antibiotics. This phenomenon may be a mechanism for ecosystem self-protection [25]. Nitrogen-cycling microorganisms play an important role in aquatic sediments and participate in the transformation and cycling of nitrogen [26]. The use of antibiotics may lead to changes in the structure and quantity of microbial communities in water bodies, thereby affecting the activity and diversity of nitrogen-cycling microorganisms.

The aim of this study was to investigate the effects of the antibiotics sulfamethoxazole (SMZ), norfloxacin (NOR) and oxytetracycline (OTC) on nitrogen-cycling microorganisms in aquaculture water. The main objectives of this study were (1) to evaluate the environmental variables (TN, NH_4^+−N, NO_3^-−N and NO_2^-−N) in overlying water and sediments; (2) to assess changes in the microbial community structure of the sediments; (3) to determine the abundance of genes (*AOA amoA*, *AOB amoA*, *nxrB* and *nosZ*) in sediments; and (4) to determine the correlation between nitrogen concentration, gene abundance and the microbial community. This study provides an in-depth understanding of the ecological effects of the antibiotics oxytetracycline, norfloxacin and oxytetracycline on the aquaculture environment and helps us better understand the potential mechanisms by which the environment responds to these stressors.

2. Materials and Methods

2.1. Chemicals and Reagents

The antibiotics SMZ (98%) and OTC (95%) were purchased from Beijing Soleb Technology Co., Ltd. (Beijing, China), and NOR (98%) was purchased from Beijing Biology Technology Co., Ltd. (Beijing, China). The other experimental drugs, including CTAB, SDS, Tris and EDTA, were purchased from Seguo Biotechnology Co., Ltd. (Zhengzhou, China), and chloroform, sodium acetate, anhydrous ethanol, chloroform mixture with phenol, sodium chloride and disodium bisulfate, all of which were of analytical purity grade, were purchased from Sinophosphoric Chemical Reagents Co., Ltd. (Shanghai, China); peptone was purchased from Tianjin Dingsheng Xin Chemical Co., Ltd. (Tianjin, China). DNA maker, IPTG, X-Gal, ampicillin and agarose were purchased from Dalian Takara (Dalian, China), and these drugs were of biochemical grade. The sediments and in situ water used in this study were collected from an aquaculture pond (30°29′53.56″ N, 114°21′2.56″ E) on the Nanhu Fishing Ground in Wuhan. Nanhu Lake is a eutrophic shallow lake located in the middle reaches of the Yangtze River in Wuhan, Hubei Province, China.

2.2. Batch Treatment

For each 1 L flask, 50 mL of sediment and 450 mL of in situ water were added, and the mixture was preincubated at 25 °C with shaking at 60 r/min for 3 days. Then, different antibiotics at the concentrations used for the Nanhu fishing grounds were added, and the plants were cultured in a shaker. The concentrations of SMZ, NOR and OTC added were 0.6, 1.05 and 3.9 mg/L, respectively, and the concentrations were doubled on the first day. The SMZ and NOR exposure groups were continuously supplemented with antibiotics for 6 days, and the OTC exposure group was continuously supplemented for 5 days. The water samples were collected on day 0 and every 24 h for a total of 14 days. The sediment samples were collected on days 0, 7 and 14. The control group was not treated with antibiotics, and three parallel experiments were performed for in each group.

2.3. Determination of the Water Quality Index

Total nitrogen was determined by potassium persulfate oxidation–ultraviolet spectrophotometry [27]. Ammonia nitrogen was determined by Nessler's reagent colorimetry [28]. Nitrate nitrogen was determined by amino sulfonic acid–ultraviolet spectrophotometry [29]. Nitrite nitrogen was determined by N-(1-naphthyl)-ethylenediamine spectrophotometry [18].

2.4. Determination of Functional Genes of the Nitrogen Cycle

Ten grams of sediment was centrifuged at 3500 r/min for 15 min, and then the precipitation was used to determine the dry weight, after which the DNA was extracted following previous methods [30]. The expressions of four functional genes, *AOA amoA*, *AOB amoA*, *nxrB* and *nosZ*, were determined via fluorescence quantitative PCR. The primers used for each functional gene are shown in Table 1.

Table 1. Primer information table for related functional genes.

Functional Gene	Name of Primers	Primer Sequence (5′ to 3′)	Ref.
AOA amoA	*CrenamoA23f* *CrenamoA616r*	ATGGTCTGGCTWAGACG GCCATCCABCKRTANGTCCA	[31]
AOB amoA	*nxrB-F* *nxrB-R*	TACATGTGGTGGAACA CGGTTCTGGTCRATCA	[32]
nxrB	*amoA-1F* *amoA-2R*	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	[33]
nosZ	*nosZ-F* *nosZ-1622R*	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	[34]

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 and a final extension at 72 °C for 5 min. PCR reactions were performed in 20 μL upstream and 0.5 μL downstream primers, 10 μL 2 × Taq Master Mix (Roche), 7.5 μL ddH_2O and 2 μL template DNA. After the PCR product was obtained, 60 μL of the PCR product was collected and mixed with the corresponding volume of buffer. The target band was cut, and the target fragment was recovered by a gel extraction kit. The purified PCR products were ligated to the pMD18-T (Bao Biological Engineering (Dalian) Co., Ltd., Dalian, China) vector by a DNA ligation kit. The above products were subsequently transferred into DH5α chemo-sensing cells, and then the plasmids were extracted from the expanded cultured strains with a TIANprep Mini Plasmid Kit. The other materials mentioned above were all purchased from Beijing Zoman Biotechnology Co., Ltd. (Beijing, China). The *AOA amoA, AOB amoA, nxrB* and *nosZ* genes were amplified, and the qPCR results were analyzed using the ABI Quant Studio 3 quantitative PCR instrument. The amplification efficiency was excellent, at 93%, 97%, 95% and 94%, respectively, which met the requirements of the standard curve.

2.5. High-Throughput Sequencing

High-throughput sequencing of the V3–V4 region was performed at Jinweizhi Biotechnology Co., Ltd. (Suzhou, China). The concentration of the library was quantified to 10 nM and the sequence information was read by the relevant software provided by the instrument. OTU clustering analysis was performed by using a similarity threshold. The rare OTUs (serial numbers <0.1 of the total serial number) were removed, and the dilution curve was checked for verification of the sampling depth. The 16S rRNA gene sequence corresponding to the functional gene of the nitrogen cycle in the NCBI was combined with the OTU annotation name and the corresponding 16S rRNA gene sequence. For community and diversity, MOTHUR (V.1.39.5) software was used to analyze the original sequence.

2.6. Data Analysis

One-way ANOVA analysis was used to compare the differences in nitrogen concentrations and functional gene abundance between the experimental group and the control group on the same day and the differences in functional gene abundance in the same group at different time points. SPSS software (Version 22, IBM, Chicago, IL, USA) was used to count, process and analyze the data, and Origin Pro 2019b was used for plotting.

3. Results

3.1. Changes in Various Nitrogen Compounds in Water

The concentrations of NH_4^+−N, NO_3^-−N, NO_2^-−N and TN in different antibiotic exposure treatments (SMZ, NOR and OTC) for 14 days are shown in Figures 1–3. There was no significant difference in the concentrations of NH_4^+−N or TN between the sulfonamide exposure group and the control group within 14 days ($p > 0.05$). The change in the NO_3^-−N and NO_2^-−N concentrations was found to have a very significant difference on the 13th day ($p < 0.05$). The concentration of NO_2^-–N began to change differently from the 10th day. The increase in NO_2^-−N in the SMZ treatment was delayed by 1 day compared with that of the control group, and then it decreased rapidly to 0.09 mg/L on the 13th day ($p < 0.05$).

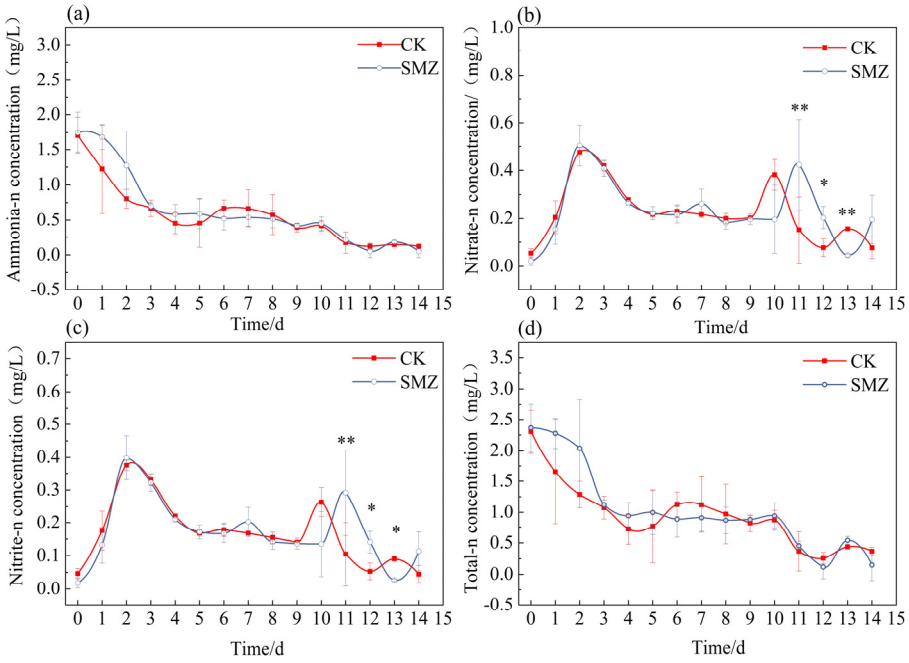

Figure 1. Temporal changes of NH_4^+–N (**a**), NO_3^-–N (**b**), NO_2^-–N (**c**), and TN (**d**) concentrations with or without SMZ treatment. * indicates $p < 0.05$, ** indicates $p < 0.01$.

NOR treatment had no significant effect on the removal of NH_4^+−N or TN, which decreased to 0.122 and 0.469 mg/L, respectively, after 14 days (Figure 2a,b). Similarly, on days 6 and 13, the NO_3^-−N concentrations in the NOR treatment group were 31% and 69% lower than those in the control group, respectively ($p < 0.05$). The two treatments eventually decreased to the same level, reaching approximately 0.076 mg/L (Figure 2b). The change in the NO_3^-−N and NO_2^-−N concentrations was found to have a significant difference on the 10th day ($p < 0.05$). In the control group, the concentrations of nitrate and nitrite increased greatly, while the concentration of ammonia nitrogen decreased to a certain extent. The concentration of nitrite nitrogen (Figure 2c) in the addition group was 13% lower than that in the control group on the 3rd day ($p < 0.05$), and concentrations were 43%, 40%, 31% and 69% lower than those in the control group on the 4th, 5th, 6th and 13th days, respectively ($p < 0.01$).

Figure 3 shows that the total nitrogen (TN) concentration in both the OTC treatment and control groups decreased to a lower level (0.629 mg/L) on the 14th day. The concentrations of NH_4^+−N in both the OTC-treated and untreated plants (Figure 3a) decreased greatly during the first two days, and then increased and fluctuated. On the 4th and 6th days, the concentrations in the OTC exposure group were 94% and 357% greater than those in the control group, respectively, and then decreased rapidly to a similar concentration to that in the control group. On the 10th day, the concentration of nitrate nitrogen increased rapidly, while the concentration of ammonia nitrogen also decreased rapidly. The NO_3^-−N concentrations in the addition group were 75%, 66% and 220% greater than those in the

control group on days 4, 5 and 13, when the NO_3^-−N concentrations were 75%, 66% and 220% greater than those in the control group, respectively (Figure 3b). The changes in the NO_2^-−N and TN concentrations in the addition group and the control group were almost the same (Figure 3c,d).

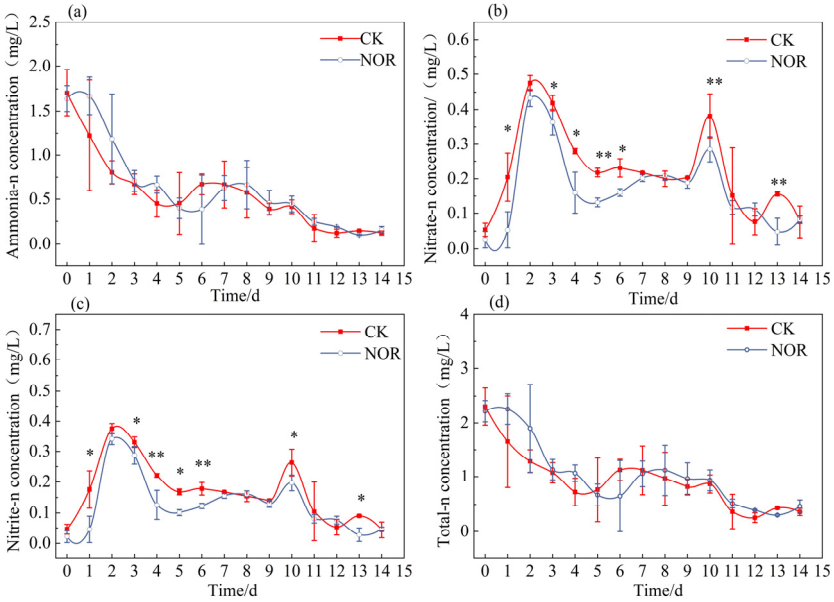

Figure 2. Temporal changes of NH_4^+–N (**a**), NO_3^-–N (**b**), NO_2^-–N (**c**), and TN (**d**) concentrations with or without NOR treatment. * indicates $p < 0.05$, ** indicates $p < 0.01$.

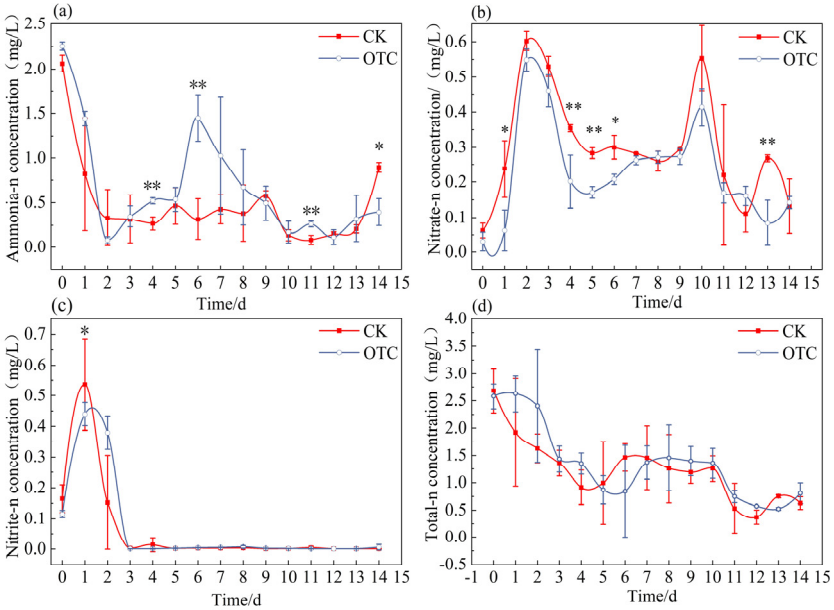

Figure 3. Temporal changes of NH_4^+–N (**a**), NO_3^-–N (**b**), NO_2^-–N (**c**), and TN (**d**) concentrations with or without OTC treatment. * indicates $p < 0.05$, ** indicates $p < 0.01$.

3.2. Nitrogen-Cycling Gene Abundance

In both the control and SMZ treatment groups, gene abundance increased significantly at 7 and 14 days compared with that at 0 days (Figure 4). There was no significant difference in the abundance of the four nitrogen functional genes between the SMZ treatment groups and the control group on days 0, 7 and 14, meaning that the SMZ treatment had no significant effect on the growth of nitrogen-cycling microorganisms at the experimental level.

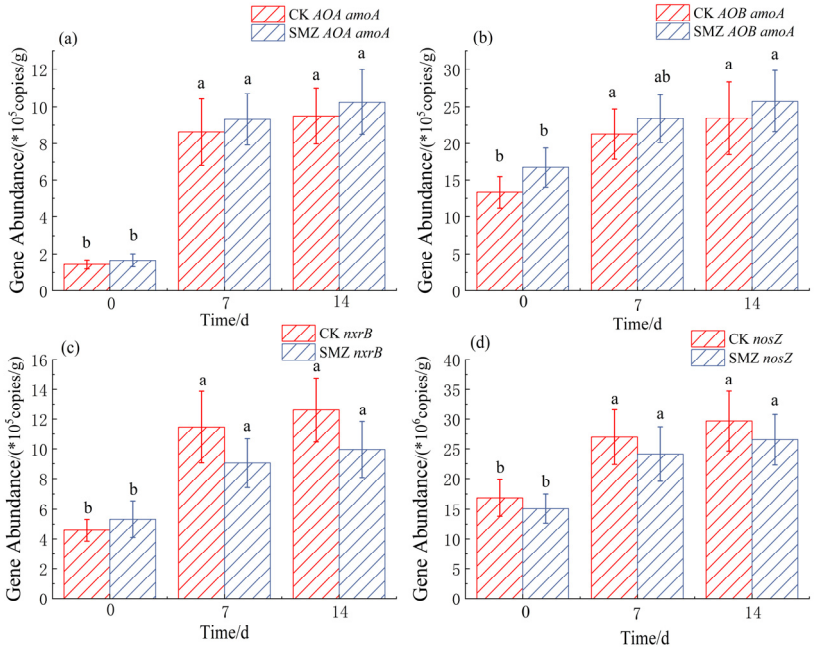

Figure 4. a,b: Abundance of nitrogen-cycling genes, including *AOA amoA* (**a**), *AOB amoA* (**b**), *nxrB* (**c**) and *nosZ* (**d**) in sediment samples with SMZ treatments.

After NOR was added, the abundance of each functional gene exhibited different degrees of inhibition (Figure 5). The abundance of *AOA amoA* in the NOR-addition and control groups increased with the increasing incubation time, and the increase in the control group was greater than that in the NOR treatment group. Similarly, compared with those on the initial day, the abundances of *AOB amoA* and *nosZ* in the NOR-treated group on day 7 decreased by 10% and 57%, respectively. Subsequently, by day 14, the abundances of *AOB amoA* and *nxrB* returned to their initial levels, while *nosZ* remained unchanged, beginning on day 7. This indicated that NOR had stronger inhibition of *nosZ* than the other three genes.

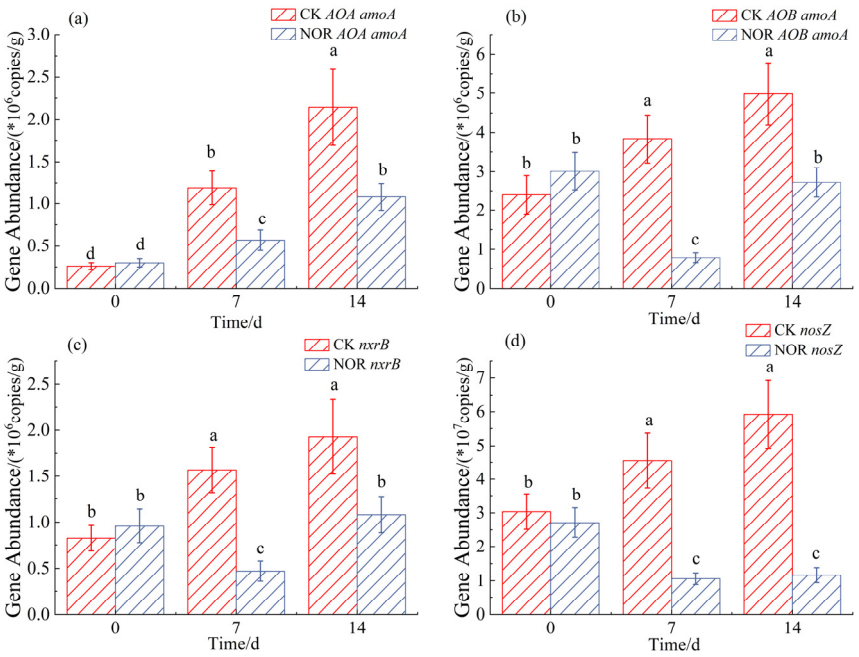

Figure 5. a–d: Abundance of nitrogen-cycling genes, including *AOA amoA* (**a**), *AOB amoA* (**b**), *nxrB* (**c**) and *nosZ* (**d**) in sediment samples with NOR treatments.

Figure 6 shows that the abundance of *AOB amoA* and *nxrB* on the 7th and 14th days in the OTC-treated group was negatively related to that on day 0. The inhibition rates reached 74% and 64% and 21% on day 7, and 78% and 84% on day 14, respectively. Unlike what was observed after the addition of NOR, the abundance of these two genes did not increase with the increasing exposure time. The *nosZ* gene had an insignificant effect on the response to OTC addition. OTC increased the abundance of the *AOA amoA* by 179% compared with that of plants not treated with OTC for 7 days, which was opposite to the findings for the other three genes and different from the effects of SMZ (no effect) and NOR (inhibition) on this gene.

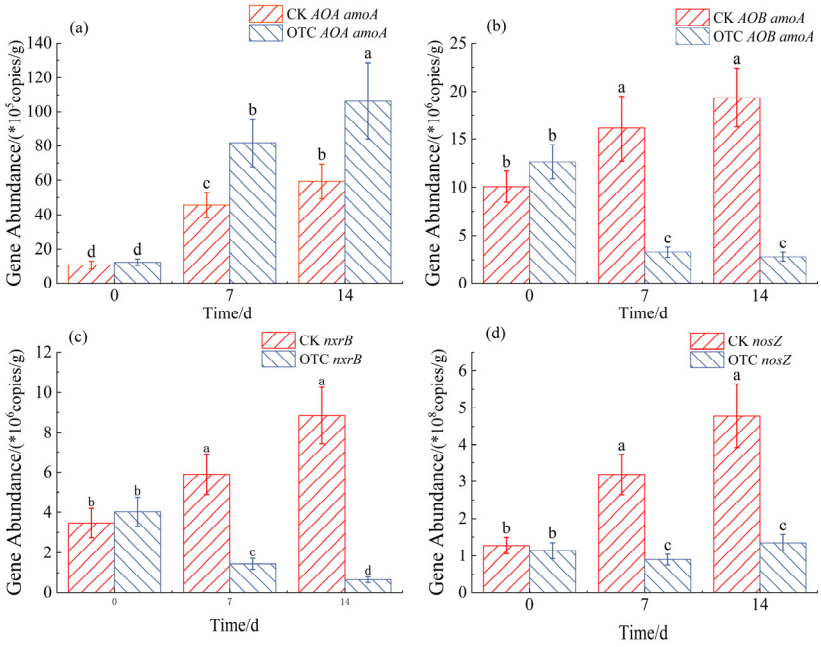

Figure 6. a–d: Abundance of nitrogen-cycling genes, including *AOA amoA* (**a**), *AOB amoA* (**b**), *nxrB* (**c**) and *nosZ* (**d**) in sediment samples with OTC treatments.

3.3. Composition of Microbiome Communities

Figures 7–9 show the effects of the three antibiotics on the microbial community compositions of aquaculture sediments at the phylum and genus levels, respectively. Figure 7 shows the specific distribution of SMZ microorganisms related to nitrification and denitrification at the phylum level. The dominant phyla were *Proteobacteria* (45.73%), *Desulfobacterota* (11.56%), *Acidobacteriota* (5.61%), *Chloroflexi* (7.84), *Nitrospirota* (6.62%), *Bacteroidota* (5.44%), *Nitrospinota* (2.70%), *Acidobacteriota* (0.33%), *Themoplasmatota* (0.31%) and *Firmicutes* (0.18%). *Proteobacteria* dominated, but its abundance decreased by 29% and 30% with or without SMZ, respectively. The trend in the abundance of *Nitrospinota* was the opposite. Compared to that in the day 0 group, the abundance of *Nitrospinota* in the control group increased by 23%, while that in the SMZ treatment group increased by 49% after 7 days. At the genus level, *Thiobacillus* (7.40%), *P9X2b3D02* (2.70%), *Bacteroidetes_vadinHA17* (2.31%), *4-29-1* (1.97%) and *Candidatus* (0.51%) were the dominant genera. The abundance of *Actinobacteriota* after 14 days in the SMZ treatment group was 33% greater than that in the control group, but the overall proportion was low and did not change significantly. Overall, SMZ had no significant impact on nitrogen-cycling microorganisms at the phylum or genus level.

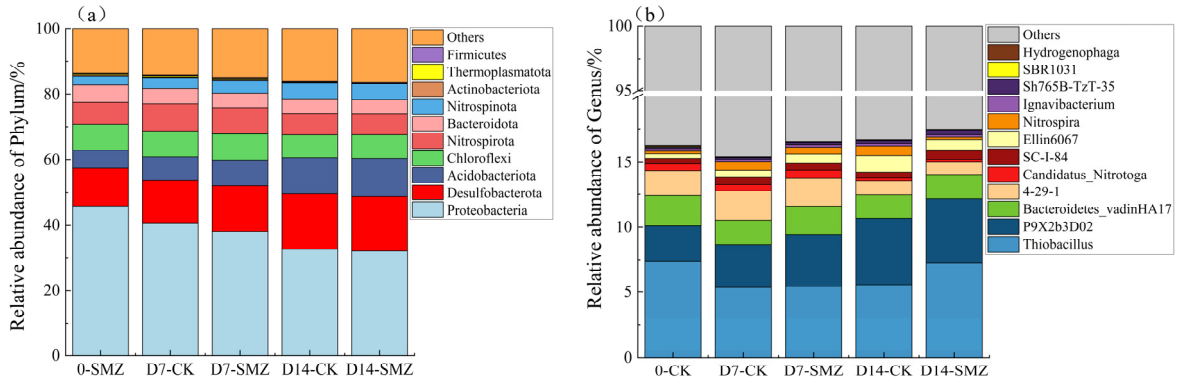

Figure 7. The relative abundances of phyla (**a**) and genera (**b**) in samples with or without SMZ treatment.

Figure 8a shows the specific distribution of microorganisms related to the nitrogen cycle at the phylum level in the NOR test, which included *Proteobacteria* (45.73%), *Desulfobacterota* (11.56%), *Chloroflexi* (7.87%), *Nitrospirota* (6.62%) and *Acidobacteriota* (5.61%). Halobacterota was the most affected by NOR. The relative abundance of *Halobacterota* in the control group decreased by 13%, while it increased by 22% with the NOR treatment. At the genus level, *Thiobacillus* (7.40%), *P9X2b3D02* (2.70%), *Sva0485* (5.64%) and 4-29-1 (1.97%) were the dominant genera (Figure 8b). *Sva0485* was significantly inhibited by NOR. On the 7th day, the relative abundance of *Sva0485* decreased by 12.77% in the NOR treatment group but increased by 14.0% in the control group.

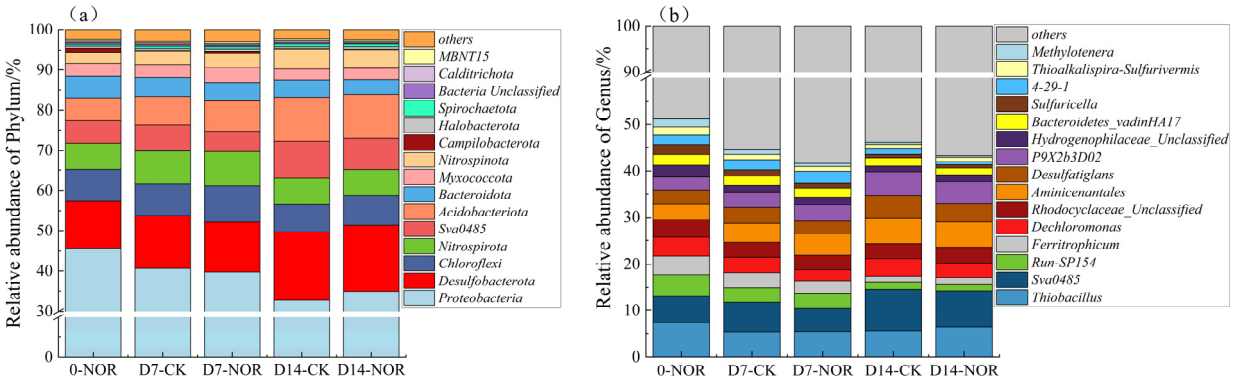

Figure 8. The relative abundances of phyla (**a**) and genera (**b**) in samples with or without NOR treatment.

Figure 9a shows the effect of oxytetracycline on nitrogen-cycling-related microorganisms at the phylum level. The dominant phylums were *Proteobacteria* (34.83%), *Firmicutes* (25.88%), *Nitrospirota* (5.10%), *Actinobacteriota* (4.81%), *Nitrospinota* (2.75%) and *Bacteroidota* (1.56%), which together accounted for more than 60% of the total OTUs. Similarly, compared with that of the control group, the abundance of *Bacteroidota* increased by 21% on the 7th day and subsequently decreased by 29% on the 14th day, and the abundance of *Actinobacteriota* was inhibited by 63%. Figure 9b shows the specific distribution of microorganisms related to the nitrogen cycle at the genus level. The dominant genera were *Thiobacillus* (6.00%), *P9X2b3D02* (2.75%), *4-29-1* (0.69%) and *SC-I-8* (0.54%). Oxytetracycline promoted the growth of *Thiobacillus* and *P9X2b3D02*, and their relative abundances increased by 26% and 28%, respectively.

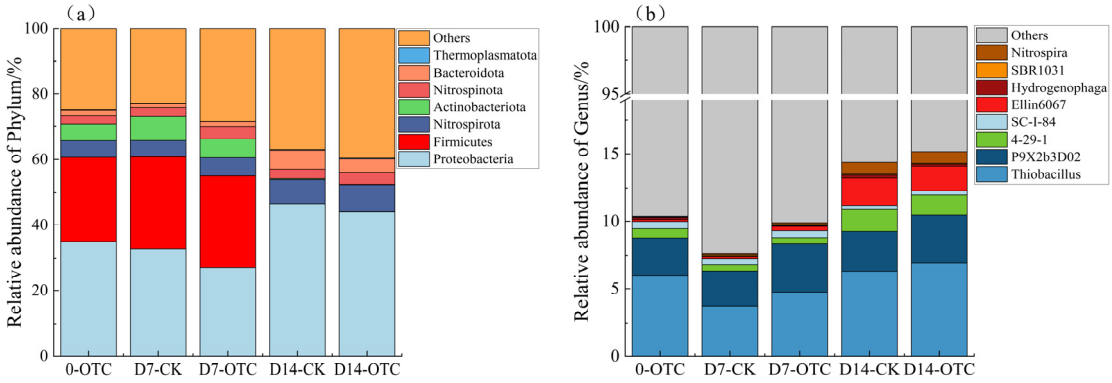

Figure 9. The relative abundances of phyla (**a**) and genera (**b**) in samples with or without OTC treatment.

4. Discussion

The experimental results showed that the effects of three different drugs, sulfamethoxazole (SMZ), norfloxacin (NOR) and oxytetracycline (OTC), on nitrogen-cycling microorganisms in the sediments of aquaculture water showed different degrees of inhibition.

SMZ exposure had no significant impact on the nitrogen levels in aquaculture water bodies, nitrogen-cycling-related functional genes or microbial abundance in the sediment. This may be explained by the fact that the amount of SMZ used in the treatment group had not yet reached a high level and may not have been enough to cause changes. The addition of antibiotics has little effect on ammonia nitrogen. Therefore, ammonia nitrogen will still decrease within the normal antibiotic dose application range. And the addition of antibiotics does not have a large effect on nitrogen metabolism. Zhang [35] reported that 200 mg/kg SMZ had a certain inhibitory effect on the bacterial community in aquaculture water, but the cumulative concentration in this work was 20 mg/kg, which was lower than that previously reported. Wang [36] also reported that 20 mg/L SMZ had no significant effect on the nitrification process of microorganisms in the first 10 days. Therefore, the exposure concentration and duration of SMZ may have led to these results. Moreover, because both the water and the sediment came from actual aquaculture ponds, the long-term use of SMZ had a certain screening effect on the microorganisms in the sediments, resulting in the presence of more antibiotic-resistant bacteria. These microorganisms may adapt better when exposed to antibiotics again. According to Naylor [37], more than 50% of aquaculture environments and aquaculture organisms have drug resistance problems.

NOR inhibited the increase in abundance of both the *AOA amoA* and the *AOB amoA* genes. The functional gene abundance increased again after the NOR test was stopped (at 7 days), after which the plants recovered for 7 days. NOR had a greater inhibitory effect on *AOB amoA*, probably due to the greater sensitivity of AOB than AOA. Pan [38] reported that the inhibitory effect of NOR on *AOB amoA* was significantly greater than that on *AOA amoA*. The inhibitory effect of NOR on *nxrB* slightly recovered on the 14th day, while that on *nosZ* did not. The inhibitory effect of NOR on *nxrB* led to a certain inhibition of nitrification, the inhibition of nitrous oxide reductase was relatively strong, and the concentrations of nitrite nitrogen and nitrate nitrogen were lower than those in the control group. Chen [39] also reported that antibiotics can reduce the activity of denitrifying enzymes and inhibit the degradation of nitrate nitrogen.

Unlike NOR, OTC only inhibited the abundance of *AOB amoA* but promoted the abundance of *AOA amoA*. Wei [40] found that a low concentration (0.05 mmol/kg) of OTC could promote the abundance of *AOA amoA* in soil, while a high concentration (0.8 mmol/kg) had the opposite effect. The concentration of OTC in this work was low, which was consistent with previous reports. Similarly, Schauss [41] reported a similar phenomenon. These authors suggested that after the function of AOB was inhibited, AOA with the same ecological function was further strengthened, thereby supplementing the functional deficiency of AOB (functional redundancy between AOA and AOB). There were

significant differences in the effects of the three antibiotics on *AOA amoA* and *AOB amoA*, indicating that ammonia-oxidizing microorganisms have different sensitivities to various antibiotics, which may be due to the degree of tolerance and adaptation of microorganisms to antibiotics [42]. OTC had the strongest inhibitory effect on *nosZ* gene abundance, which was consistent with the findings of previous studies. The *nosZ* gene encodes nitrous oxide reductase, whose abundance reflects the level of denitrification activity [43]. Zou [14] also reported that OTC can significantly decrease the abundance of the *nosZ* gene in sediment within 16 days and reduce denitrification characteristics.

Analysis of the sediment microbiome communities revealed that *Proteobacteria* was the main phylum in each sample [44], which was consistent with the distribution of bacterial communities in various river and lake sediments [45]. Proteobacteria include phototrophic, chemoautotrophic and chemoheterotrophic bacteria, which are ubiquitous in aquatic environments due to their wide adaptability and rapid growth [46]. At the genus level, *Sva0485* was the most inhibited by NOR. *Sva0485* is a nitrite-reducing bacterium that can reduce nitrite to nitrogen. Under NOR stress, the abundance of *Sva0485* showed the same decreasing trend as the abundance of the *nosZ* (Figures 5d and 8). The abundance of heterotrophic bacteria (such as nitrifying bacteria) increased, thereby reducing the concentration of nitrite nitrogen [47].

Before and after the addition of oxytetracycline, the relative abundance of nitrogen-cycling microorganisms at different phylum levels in the system changed. *Proteobacteria* were widely distributed in each sample site with an absolute advantage, and the relative abundance was greater than 40%. The effect of oxytetracycline on the sediment's microbial *Proteobacteria* showed a significant increase in relative abundance on the 14th day. Studies have shown that the abundances of *Proteobacteria* and *Bacteroidetes* are closely related to the concentrations of nitrate, nitrite and ammonia. *Proteobacteria* is the most abundant *phylum* in denitrification systems and is associated with different carbon sources and ecological conditions in water. *Bacteroidetes* can biodegrade macromolecules such as proteins, starch and cellulose, supporting the hydrolysis and utilization of organic matter during denitrification. The structure and activity of microbial communities often change under antibiotic stress. Studies have confirmed that *Proteobacteria* play an important role in biological nitrogen removal. *Proteobacteria* include major nitrogen-removal functional bacteria, such as nitroso bacteria, nitrifying bacteria and denitrifying bacteria [48,49]. Under oxytetracycline stress, these bacteria may develop resistance or acquire resistance genes through horizontal gene transfer [50,51]. Yang [52] reported that the use of tetracycline antibiotics inhibited the growth of soil bacteria and actinomycetes, significantly reduced the soil microbial biomass and promoted an increase in soil fungi. The reason for the inhibition of nitrogen-cycling microorganisms in this study may be that the concentration of OTC used was lower than that used in other studies, and a low concentration of oxytetracycline had little effect on the number of bacteria and may also promote the formation of drug-resistant bacteria; therefore, relatively speaking, the growth of bacteria was inhibited. This inhibitory effect was significantly enhanced with increasing the OTC treatment dose and did not disappear during the 30-day culture period.

5. Conclusions

This work investigated the effects of SMZ, NOR and OTC on the conversions of various nitrogen-cycling genes in aquaculture water and the abundance of nitrogen-cycling microorganisms in sediments. SMZ exposure had no significant impact on the nitrogen levels in aquaculture water bodies, nitrogen-cycling-related functional genes or the microbial abundance in the sediment. NOR inhibited the abundance of four nitrogen-conversion genes. Except for NosZ, the abundance of the other three genes recovered after the discontinuation of NOR treatment. Unlike NOR, OTC only inhibited the abundance of *AOB amoA*, while it promoted the abundance of *AOA amoA* due to the functional redundancy between AOA and AOB. The inhibition of *AOA amoA*, *nxrB* and *NosZ* by OTC could not be restored after stopping the exposure. Moreover, through the changes in relative abundance

at the phylum and genus levels, the nitrogen cycle-related bacterial phylums in the SMZ experimental group and the control group remained consistent. The gene abundance of the denitrifying bacterium *Sva0485* in the NOR group decreased by 12.77%. The survival of Firmicutes, the dominant bacteria in the OTC group, was completely inhibited, and the denitrification of nitrogen was inhibited. Various antibiotics had different sensitivities to the microorganisms, which related to the relative abundance of nitrogen-conversion genes and related microorganisms. The presence of native drug-resistant bacteria in the in situ aquaculture water and sediment may have reduced the stimulation intensity after re-exposure to antibiotics. Although the use of antibiotics in aquaculture has little impact on the removal of various nitrogen-cycling genes, it may increase the antibiotic adaptability of microbial communities and pose potential risks to human health. Therefore, standardized use of antibiotics in aquaculture is particularly important.

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