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Effect of the Sulfamethazine on Nitrogen Conversion in Alternate Wet and Dry Paddy Fields

Lina Chen [1,2,3,4], Haiming Wang [1,2,3], Jiang Li [1,*], Wenbo Wu [1,2,3] and Guomian Guo [1,4]

1 College of Agricultural Science and Engineering, Hohai University, Nanjing 211100, China
2 Key Laboratory of Integrated Regulation and Resource Development on Shallow Lake of Ministry of Education, Hohai University, Nanjing 211100, China
3 College of Environment, Hohai University, Nanjing 210098, China
4 Key Laboratory of Lower Yellow River Channel and Estuary Regulation, Ministry of Water Resources, Zhengzhou 450003, China
* Correspondence: lijiang@hhu.edu.cn

Abstract: Aquaculture wastewaters have been used in rice irrigation directly or indirectly. Antibiotics in aquaculture wastewater entering the rice fields with irrigation will affect the soil physicochemical properties, microbial properties, and nitrogen conversion. A pot experiment irrigated with simulated aquaculture wastewater was performed to study the effects of sulfamethazine in aquaculture wastewater on nitrogen concentration and conversion-related microorganisms in rice fields with different irrigation modes. Sulfamethazine (500 ng/L, 1500 ng/L, and 3000 ng/L) decreased the NH_4^+ concentration at the late tillering stage and NO_3^- concentration at the late tillering and jointing–booting stages ($p < 0.05$) but increased the NH_4^+ concentration at the late tillering stage ($p < 0.05$). Sulfamethazine (3000 ng/L) promoted the lowest nitrogen conversion gene (*amoA*, *nirS*, and *nirK*) abundances and the most special community structure of nitrogen conversion microorganism under mild alternate wetting and drying (AWD). Furthermore, *Nitrosospira_sp._KAN8*, belonging to ammonia-oxidizing bacteria (AOB), was sensitive to sulfamethazine. Flora with the same nitrogen conversion genes exhibited different variations under the same treatment. The results show that antibiotic and mild AWD caused more serious adverse effects to soil nitrogen conversion and nitrogen conversion microorganisms, which will increase the environmental risks of sulfamethazine. It can provide a basis for the scientific and rational use of aquaculture water to irrigate rice fields.

Keywords: alternate wetting and drying irrigation; antibiotic; Illumina sequencing; nitrogen conversion microorganism; PCR

Citation: Chen, L.; Wang, H.; Li, J.; Wu, W.; Guo, G. Effect of the Sulfamethazine on Nitrogen Conversion in Alternate Wet and Dry Paddy Fields. *Agronomy* **2022**, *12*, 3034. https://doi.org/10.3390/agronomy12123034

Academic Editor: Salvatore Camposeo

Received: 25 October 2022
Accepted: 26 November 2022
Published: 30 November 2022

1. Introduction

The global overuse of ground and surface water resources has caused serious environmental problems [1]. Due to greenhouse gas emissions, droughts of the future are likely to be more frequent, severe, and longer-lasting than they have been in recent decades [2]. Water scarcity is increasingly becoming a key constraint on global development. To obtain more available water resources, the development and utilization of unconventional water resources such as wastewater has attracted more and more attention from various countries [3,4]. Agriculture consumes the most water resources, agricultural water consumption accounts for nearly 60% of the total water consumption in China, and irrigation consumes 90% of the total agricultural water consumption. In the meantime, unconventional water resources such as aquaculture wastewater contain the nitrogen and phosphorus required for crops. Therefore, the usage of unconventional water resources for irrigation has important prospects and significance in coping with water shortages [5].

Aquaculture wastewater, one of the common unconventional water resources, is rich in nutrients such as nitrogen and phosphorus owing to excreta and unused feed [6]. Its application to irrigation is an effective means to achieve "solving aquaculture pollution

emissions and alleviating irrigation water shortages" [7,8]. However, some new pollutants, such as antifungal compounds, hormones, and antibiotic, are produced in aquaculture production [9–11]. These pollutants will be a potential contributor to farmland pollution. Residual antibiotics are one of the most typical pollutants. They have been widely detected in natural water bodies, which is mainly sulfamethazine [12–15]. The concentration of antibiotics in water is often less than 3200 ng/L [12,16].

Antibiotics are used in animal husbandry as antimicrobial growth promoters to control infectious diseases and accelerate animal growth. Antibiotics used in animals are not fully absorbed. They are excreted and enter the farmland with fertilization or irrigation [17,18]. Antibiotics are widespread in the water and soil [19,20], and it has been found that the concentration of sulfonamide antibiotics in agricultural soils is 0–27.93 μg/kg [21], and the average concentration is 6.92 μg/kg [22]. A study along the Mekong Delta in Vietnam found that the highest sulfamethazine concentration was 86.1 μg/kg [23] in rice field soils. Previous studies have shown that antibiotics in soils can not only lead to changes in the community structure of soil microbes [24,25] but also have an impact on the nitrogen conversion process [26–29].

Aquaculture ponds and rice fields are staggered in the plain lake areas of South China, and aquaculture wastewater has been used directly or indirectly for irrigation [30]. At the same time, rice has different irrigation methods, such as flood irrigation and alternate wetting and drying (AWD) [31]. AWD reduces water consumption and changes the long-term anaerobic environment of flood-irrigated rice fields. It leads to changes in the soil physicochemical characteristics of rice fields [32] and will cause differences in soil antibiotic residues. These changes will lead to differences in soil microbes and nitrogen conversion, whereas very few studies have examined this.

The objective of this study was to reveal the effects of antibiotics on soil nitrogen and nitrogen conversion microorganisms in paddy fields under different intensities of AWD to study the adverse effects of antibiotics on rice fields in aquaculture wastewater and ways to reduce their adverse effects. We hypothesized that AWD would promote antibiotic degradation and thus mitigate the effects on soil nitrogen conversion in paddy fields. This study can provide a basis for the scientific and rational use of aquaculture water to irrigate rice fields.

2. Materials and Methods

2.1. Experimental Design

The experiment was performed in the water-saving park of Hohai University, Jiangning District, Nanjing. The soil was taken from a paddy field in Nanjing, and it is loam. It was mixed well before experiment and passed through a coarse sieve (5 mm). The rice cultivar is Nanjing 46 (*Oryza sativa* L.), and the whole growth period is 167 days. The rice was transplanted on July 10, 2019 and harvested on 8 October 2019. A pot experiment was used. The soil pots used in the experiment were 60 cm high and 30 cm in diameter, 22 kg soil was filled inside each pot in layers, and three plants were planted in each pot. Two factors, antibiotic concentrations (A) and irrigation methods (W), were set: (1) The antibiotic concentration is the concentration of sulfamethazine in irrigation water, and 4 gradients were set, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). (2) Three irrigation methods were set, including flood irrigation (W1, except for the midseason drainage and preharvest drain, the shallow water layer was maintained during the experiment period), mild AWD (W2, each irrigation brought field water depth to 20 mm, and the next irrigation flooding is 3 days later than the disappearance of the field water), and severe AWD (W3, each irrigation brought field water depth to 20 mm, and the next irrigation flooding is 5 days later than the disappearance of the field water). There were 12 treatments, with three replicates every treatment (Table 1). The irrigation water was artificial simulated aquaculture wastewater, and the formula was: $C_6H_{12}O_6$: 33.3 mg/l, NH_4Cl: 68.76 mg/L, $NaHCO_3$: 71.4 mg/L, $NaHPO_4$: 18.86 mg/L, and mix trace elements 1 ml/L. Mixed trace elements: $MnSO_4 \cdot H_2O$: 33.8 mg/L, H_3BO_3: 49.4 mg/L,

$ZnSO_4 \cdot 7H_2O$: 43.1 mg/L, $FeSO_4 \cdot 7H_2O$: 97.3 mg/L, and $CuSO_4 \cdot 5H_2O$: 25.0 mg/L [33]. During the experiment, the precipitation was isolated.

Table 1. Processing numbers.

Treatments	Antibiotic Concentrations (A)	Irrigation Methods (W)
A0W1	A0	W1
A0W2	A0	W2
A0W3	A0	W3
A1W1	A1	W1
A1W2	A1	W2
A1W3	A1	W3
A2W1	A2	W1
A2W2	A2	W2
A2W3	A2	W3
A3W1	A3	W1
A3W2	A3	W2
A3W3	A3	W3

The base-fertilizer was 750 kg/hm^2 of compound fertilizer according to the nitrogen application level in the local area, the nutrients of the compound fertilizer were $N:P_2O_5:K_2O$ = 15:15:15, and the topdressing was 300 kg/hm^2 of urea. Topdressing was applied about 60 days after transplanting (at the jointing–booting stage). Agronomic measures such as weeding were carried out in accordance with local customs.

2.2. Soil Sampling

Soil samples were collected regularly combined with irrigation and fertilization at various growth stages of rice, the sampling depth was 5–10 cm, which was taken every 5–7 days, and the measurement was added after fertilization. Then, soil samples were kept at −20 °C in a freezer until analysis for soil nitrogen and functional genes.

2.3. Measurement of Soil Nitrogen

Soil NH_4^+ and NO_3^- were extracted with 50 mL of 0.5 M K_2SO_4 from 5.0 g fresh soils and determined using a continuous flow spectrophotometer (Auto Analyzer 3-AA3, Seal Analytical, Norderstedt, Germany).

2.4. DNA Extraction, PCR, and Sequencing of the Nitrogen Conversion Functional Genes

According to the supplier's instructions, the DNA of each sample was extracted from soil samples (A0W2, A3W1, A3W2, and A3W3) at the late tillering and yellow ripening stages, respectively, using the E.Z.N.A.® stool DNA Kit (Omega Bio-tek, Norcross, GA, USA). The quality of DNA was determined using 1% agarose gel electrophoresis and spectrophotometry. All extracted DNA samples were stored at −20 °C for the subsequent analysis.

The *amoA*, *nirS*, and *nirK* are important genes in nitrification and denitrification. AOA *amoA* and AOB *amoA* are involved in the first, often limiting, step of nitrification (oxidation of ammonium into nitrite) [34]; *nirK* and *nirS* encode nitrite reductases [35]. Referring to the methods of Liao et al. [36], we used specific primers for AOA *amoA* [37], AOB *amoA* [38], *nirK*, and *nirS* [39] to quantify the samples individually using the fluorescence PCR technique. The sequences of these primers are show in Table 2. During PCR amplification, $R^2 > 0.98$, and the amplification efficiency > 92%.

The Illumina Hi-seq (Allwegene, Beijing, China) platform was used to target ammonia oxidizing archaea (AOA) *amoA*, AOB *amoA*, *nirS*, and *nirK* genes perform high-throughput sequencing. The raw sequencing data were filtered and pruned using the PReprocessing and INformation of SEQuence data (PRINSEQ) and Moetur tools. The UCLUST tool in Quantitative Insights Into Microbial Ecology (QIIME 2) was used to conduct a cluster

analysis on sequences with a sequence similarity threshold of 97% and calculate the Alpha Diversity Index (including Shannon and Chaol) for each set of samples [40].

Table 2. Primer information used in the experimental procedure.

Genes	Name of Primer	Sequence (5′ to 3′)
AOA *amoA*	Arch-amoA26F	GACTACATMTTCTAYACWGAYTGGGC
	Arch-amoA417R	GGKGTCATRTATGGWGGYAAYGTTGG
AOB *amoA*	amoB-F	GGGGTTTCTACTGGTGGT
	amoB-r	CCCCTCKGSAAAGCCTTCTTC
nirK	nirKF1aCu	ATCATGGTSCTGCCGCG
	nirKR3Cu	GCCTCGATCAGRTTGTGGTT
nirS	cd3aF	GTSAACGTSAAGGARACSGG
	R3cd	GASTTCGGRTGSGTCTTGA

2.5. Statistical Analysis

The data were analyzed and plotted using StataMP 16.0 (StataCrop, College Station, TX, USA) and Origin 2021 (OriginLab Corporation, Northampton, MA, USA). The Kruskal–Wallis method was used for ANOVA, and Dunn's test was used for paired comparisons. The Pearson correlation analysis was performed.

3. Results

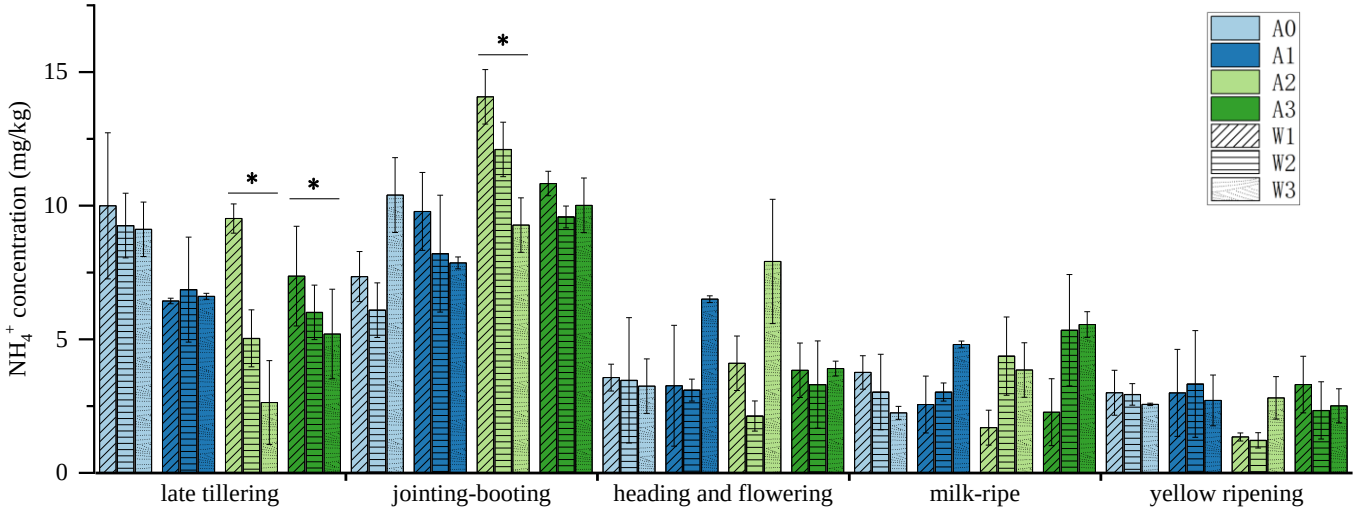
3.1. NH_4^+ and NO_3^- Concentration at Each Stage

NH_4^+ and NO_3^- concentrations are shown in Figure 1. The NH_4^+ concentration decreased with changes of the rice growth stages ($p < 0.05$), but the NO_3^- concentration of different growth stages varied less. The NH_4^+ concentration decreased with the increase of AWD intensity at the late tiller stage and heading and flowering stage ($p < 0.05$) but did not change significantly under different irrigation methods at other stages and even increased with the increase of AWD intensity ($p < 0.05$) at the milk ripe stage (Figure S1). The NO_3^- concentration showed significant differences under different irrigation methods ($p < 0.05$) at all stages, except the jointing–booting stage (Figure S2). The NO_3^- concentration decreased with the increase of AWD intensity in the late tillering period ($r = -0.4313$, $p < 0.05$), but the AWD significantly promoted the NO_3^- concentration in the other three periods. Significant differences under different sulfamethazine treatments were shown at the late tillering and the jointing stages ($p < 0.05$). Sulfamethazine significantly reduced the soil NH_4^+ concentration at the late tillering stage but significantly increased the soil NH_4^+ concentration at the jointing–booting stage. At the late tillering and jointing–booting stages, sulfamethazine significantly reduced the NO_3^- concentration.

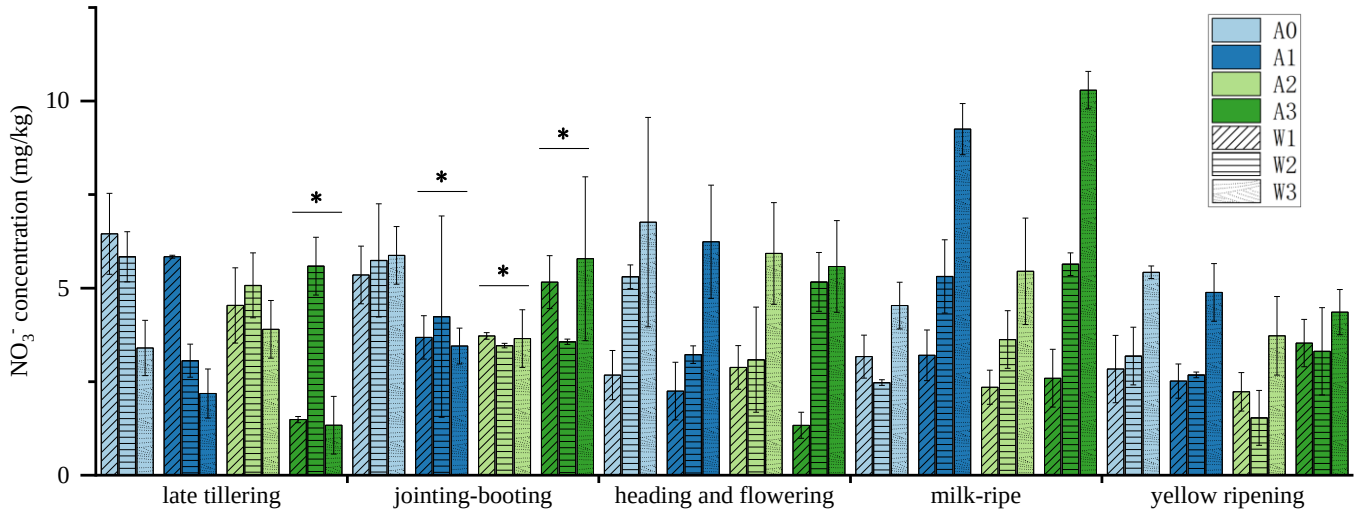
3.2. Absolute Abundance and Diversity of Nitrogen Conversion Genes

Based on the NH_4^+ and NO_3^- concentrations, the late tillering stage was selected as the representative period with a significant response to sulfamethazine, and the yellow ripening stage was selected as the representative period without a significant response to sulfamethazine for further study. During these two stages, A3 treatments with significant differences in the NH_4^+ and NO_3^- concentrations were selected, and the microbial indicators were extracted and determined. As shown in Figure 2, there were large differences between the absolute abundances of the nitrogen conversion genes. The abundance of the AOA *amoA* and AOB *amoA* were smaller. The minimum abundance of the *nirK* gene was above 4×10^7 copies/g soil, much higher than other genes. Apart from AOA *amoA*, all gene abundances showed smaller difference at the yellow ripening stage than that at the late tillering stage, while the genes in A3W2 showed lower absolute abundances compared to the other A3 treatments at the late tillering or yellow ripening stages. At the

yellow ripening stage, the AOA *amoA* appeared with lower abundance in A3W2, while the abundances of AOB *amoA*, *nirS*, and *nirK* were similar to that of other A3 treatments. In summary, A3W2 had the lowest AOA *amoA* and AOB *amoA* abundances at the late tillering and yellow ripening stages and the lowest *nirS* and *nirK* abundances at the late tillering stage. The lowest nitrogen conversion gene abundance of A3W2 attests to the special NH_4^+ and NO_3^- concentrations exhibited at A3W2 over multiple stages.



(**a**)



(**b**)

Figure 1. Soil (**a**) NH_4^+ and (**b**) NO_3^- concentrations during different growth stages. * indicates a significant difference from the A0 treatment in the same stage. A, the concentration gradient of the antibiotic sulfamethazine, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). W, irrigation methods, including W1 (flood irrigation), W2 (mild AWD), and W3 (severe AWD).

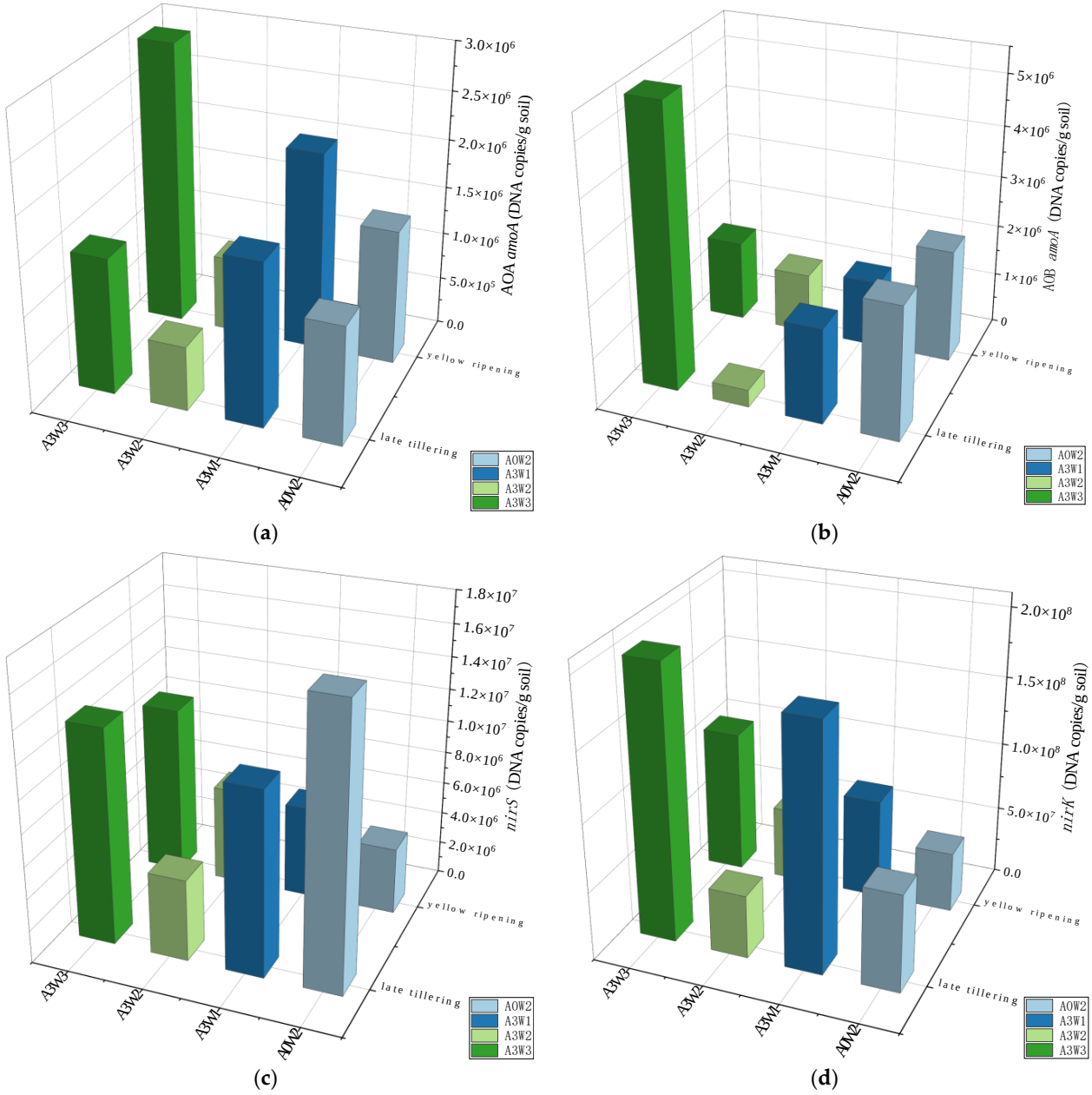


Figure 2. Absolute abundance of AOA *amoA* (**a**), AOB *amoA* (**b**), *nirS* (**c**), and *nirK* (**d**). A, the concentration gradient of the antibiotic sulfamethazine, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). W, irrigation methods, including W1 (flood irrigation), W2 (mild AWD), and W3 (severe AWD).

3.3. Alpha Diversity of Nitrogen Conversion Genes Communities

The Chao1 and Shannon diversity indices were used to study the differences in the microbial community structure under different treatments. The gene (AOA *amoA*, AOB *amoA*, *nirS*, and *nirK*) diversity indices are shown in Table 3.

The trends of the chao1 and Shannon indices were the same basically. The Shannon index of the AOB *amoA* had the greatest difference under different conditions, while the Shannon indices of other genes were smaller. The lowest Shannon index also appeared in the AOB *amoA*, while the *nirK* gene generally had a higher Shannon index. At the same time, the AOB *amoA* gene in the A0W2 had the highest Shannon index compared with others at the yellow ripening stage.

Table 3. Nitrogen conversion gene's observed Chao1 and Shannon indices.

Growth Stages	Treatments	AOA *amoA*		AOB *amoA*		*nirS*		*nirK*	
		Chao1	Shannon	Chao1	Shannon	Chao1	Shannon	Chao1	Shannon
late tillering	A0W2	24	3.113744	24	0.768873	34	2.060788	299.4286	4.729482
	A3W1	23	3.065076	19	1.155675	22	2.764163	284.6364	4.776766
	A3W2	23	2.904411	15	0.857637	17.5	2.110334	292.5526	4.368037
	A3W3	24	2.687998	21.33333	3.391534	19.5	3.050141	255.45	5.180928
yellow ripening	A0W2	24	3.129178	22	0.240285	20.2	3.707995	327	3.452603
	A3W1	23.5	3.306445	13	0.392306	20	2.956571	278.0882	4.347746
	A3W2	21	3.279984	13	0.478024	42.46154	2.113301	207.2	2.755407
	A3W3	24	3.206926	11	1.74004	18	3.458921	242	5.106296

Note: A, the concentration gradient of the antibiotic sulfamethazine, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). W, irrigation methods, including W1 (flood irrigation), W2 (mild AWD), and W3 (severe AWD).

3.4. Community Structure of Nitrogen Conversion Genes

3.4.1. Community Structure of AOA *amoA* and AOB *amoA*

A total of 30 OTUs were detected, of which the species detected were only able to annotate to the level of the classes in all treatments, and three phyla were identified except for the bacteria that failed to be annotated. Figure 3a,b shows a histogram of the phyla level and the classes level. The structure of the AOA *amoA* community changed little in all the treatments. The highest relative abundance of Crenarchaeota appeared in the A3W3_L, and the highest relative abundance of Unspecified_Archaea appeared in A3W2_Y. With the change of the growth stages, the composition of AOA *amoA* in the phyla and classes level underwent some changes. Crenarchaeota with relative abundances above 75% at the late tillering stage decreased below 75% at the yellow ripening stage (A3W2_Y slightly above 75%), while the relative abundances of Unspecified_Archaea and Thaumarchaeota increased with changes of the rice growth. In A3 treatment, Crenarchaeota had a higher relative abundance at the yellow ripening stage, while other phyla were inhibited. AWD also promoted Crenarchaeota while inhibiting other phyla, although this change may be affected by the growth period. Unspecified_Archaea has the highest relative abundance in A0W2_Y.

A total of 57 OTUs were detected in AOB *amoA*, and a total of three phyla were annotated. The relative abundance of the phyla and genera levels are shown in Figure 3c,d. The relative abundance of Proteobacteria was the highest in A0W2_L. The relative abundance of Proteobacteria in A3W2_Y was the lowest (70.2%), but the relative abundance of Proteobacteria in A3W1_Y and A3W3_Y were above 99.5%. At the late tillering stage, the relative abundance of Proteobacteria in A3 was reduced compared with A0W2_L, while the relative abundance of the other phyla increased. At the yellow ripening stage, except for A3W2_Y, antibiotics improved the relative abundance of Proteobacteria compared with A0W2_Y (more than 99.2%). The abundance distribution at the genera level is similar to that at the phyla level. *Nitrosospira* was the most abundant at the genera level, and the presence of sulfamethazine increased the abundance *of Nitrosospira* at the late tillering stage, but the abundance of *Nitrosospira* at the yellow ripening stage decreased. These changes varied due to the difference in the AWD intensity. A3W2_Y had the highest *environmental_samples_environmental_samples* abundance and the lowest *Nitrosospira* abundance of all sulfamethazine treatments. A0W2_Y had the most uniform species composition at the species level and was related to the diversity of nitrogen conversion genes in the preceding article. *Nitrosomonas_sp._Nm86* had the highest abundance at the species level.

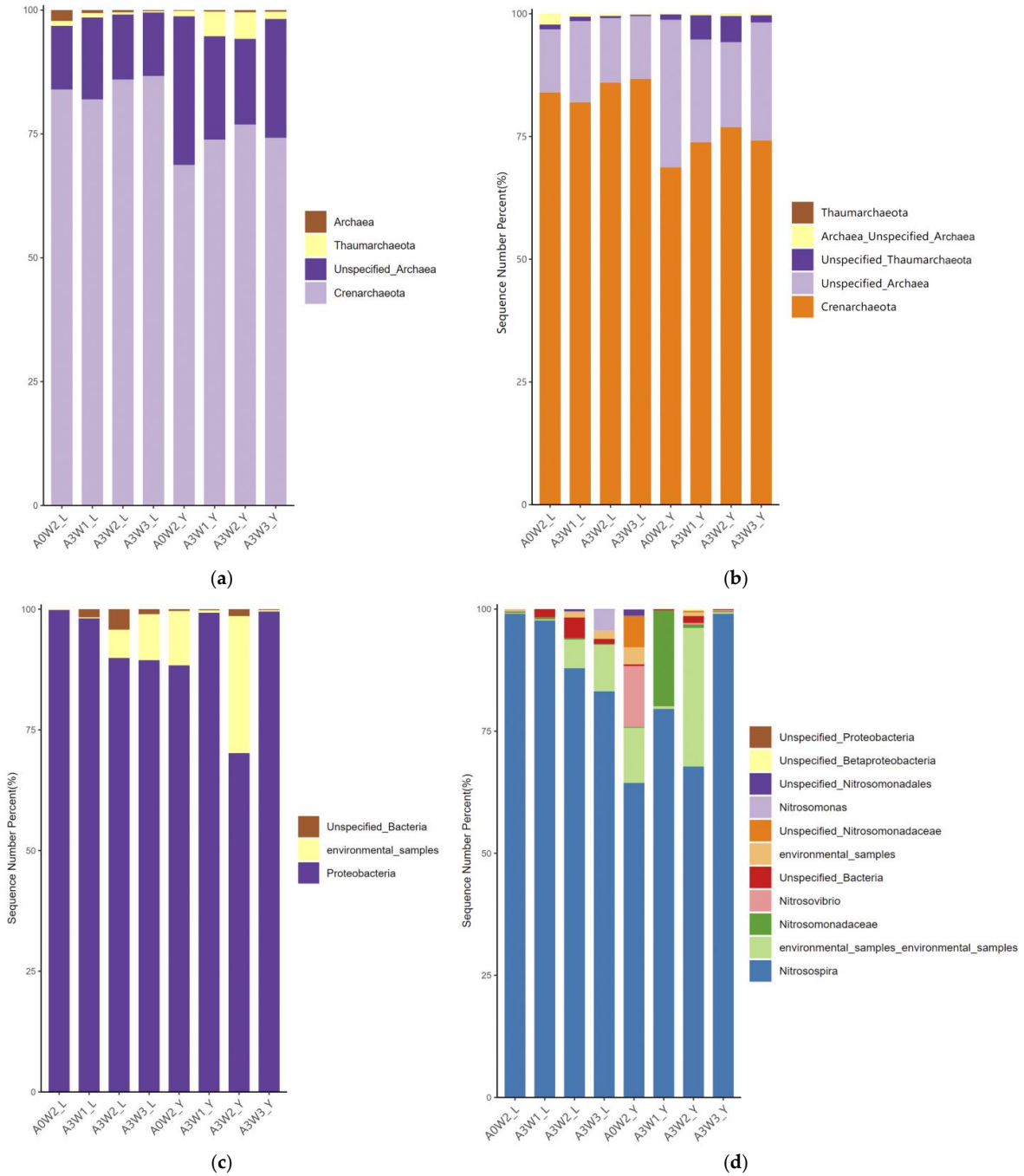


Figure 3. Relative abundance of AOA *amoA* (**a**,**b**) and AOB *amoA* (**c**,**d**) at the phyla, classes, and genera levels. A, the concentration gradient of the antibiotic sulfamethazine, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). W, irrigation methods, including W1 (flood irrigation), W2 (mild AWD), and W3 (severe AWD). _L and _Y indicate the late tillering and yellow ripening periods, respectively.

3.4.2. Community Structure of *nirS* and *nirK*

A total of 77 OTUs of the *nirS* were detected in all treatments. Five phyla were detected, and Figure 4a,b shows the community structure at the phyla and genera levels. Proteobacteria was the most abundant of all treatments, with only Proteobacteria included in A3W2_L and A3W3_L. Other phyla emerged in A3W2_Y and A3W3_Y, including Planctomycetes, Actinobacteria, and Acidobacteria. With the change of the growth stages, Acidobacteria was replaced by Chloroflexi in A0W2, Planctomycetes and Acidobacteria

were replaced by Actinobacteria in A0W3. At the genera level, the relative abundance of the microorganisms under different treatments varies greatly. The abundance distribution of *Noviherbaspirillum* had a large range different from other gene-based strains, and with the highest relative abundance always occupying higher abundances, *Noviherbaspirillum* had a relative abundance of only 3.8% in A3W3_L. At the same time, the relative abundance of *Unspecified_Burkholderiales* became the largest relative abundance in the treatment (40.8%), and the relative abundance of others also increased. Similarly, the relative abundance of *Noviherbaspirillum* in A3W1_L was only 29.8%, while the relative abundance of *Sandaracinus* reached 60.7% and became the most relatively abundant phylum in this treatment. A3W2_L had the highest *Noviherbaspirillum* abundance in the late tillering stage, while the other sulfamethazine treatments were low. In addition, with the change of the growth stage, the composition of the genera-level community structure also underwent succession, and *Noviherbaspirillum* became the genus with the largest relative abundance in A3W1 at the yellow ripening stage (92.2%). The relative abundance of *Noviherbaspirillum* in A3W3 rose to 37.4%, but the largest relative abundance of bacteria was *Pseudodogulbenkiania* (39.0%). With the changes of the growth stages, the relative abundance of *Noviherbaspirillum* decreased to a certain extent in A0W2, but the relative abundance of other bacteria increased.

A total of 450 OTUs were detected in the *nirK*, all of which belong to three phyla. Figure 4c,d shows the community structure at the phyla and genera levels. Of all treatments at the late tillering stage, A0W2_L had the highest relative abundance of Proteobacteria. The relative abundance of Proteobacteria gradually decreased with the increase of the AWD intensity in the A3 treatments. Of all the treatments at the yellow ripening stage, A0W2_Y had the lowest relative abundance of Proteobacteria, while the A3 treatments had a higher Proteobacteria abundance than A0W2_Y. At the phyla level, A3W2_Y had the lowest Proteobacteria abundance of all A3 treatments; A3W2_L and A3W2_Y had the lowest *Nitrosospira* abundances at the genera level. The different community structures of A3W2_Y may be related to the special values of the NO_3^- concentration. The genus with the highest level of abundance was generally *Nitrosospira*, and sulfamethazine decreased the abundance of *Nitrosospira* at the late tillering stage, while the relative abundance of other species increased. The relative abundance of *Nitrosospira* was higher under the A3 treatment at the yellow ripening stage, but the relative abundance of *Nitrosospira* in A3W2_Y was lower.

3.5. Correlation Analysis

As shown in Figure 5, most bacteria did not show a significant correlation with sulfamethazine. *Nitrosospira_sp._KAN8*, belonging to AOB *amoA*, can still be found with a significant negative correlation with sulfamethazine ($p < 0.001$). This suggested that both sulfamethazine-sensitive and -insensitive bacteria are present in the nitrogen conversion microorganism and that irrigation water containing sulfamethazine may have a huge impact on these antibiotic-sensitive bacteria. Except for *nirK*, other genes were not significantly associated with the irrigation methods, and *Bradyrhizobium_sp._s23321*, *Ochrobactrum*, and *Bradyrhizobium_sp._STM_3843* showed a negative correlation to irrigation ($p < 0.05$). The microorganisms of the four nitrogen conversion genes all responded to changes in the growth period of rice. Thaummarchaeota and Crenachaeota belonging to AOA *amoA* showed a diametrically opposed correlation with the growth periods ($p < 0.01$), indicating that some microbes may have adapted to the antibiotic-containing environment and underwent ecological niche changes. Other nitrogen conversion gene microorganisms correlated with the growth stage were *Janibacter_indicus*, *Pseudogulbenkiania_sp._NH8B*, and *Bradyrhizobium_diazoefficiens* positively ($p < 0.05$), and *Nitrosospira_sp._En13*, *Rhodopseudomonas_palustris*, and *Rhodopseudomonas_sp._2_8* were correlated with the growth stages negatively ($p < 0.05$). The strains of various nitrogen conversion-related genes showed a certain correlation with the NH_4^+ and NO_3^- concentrations. Among them, Crenachaeota, Archaea, *Rhodopseudomonas_palustris*, and *Rhodopseudomonas_sp._2_8* were correlated with the NH_4^+ concentration negatively ($p < 0.05$). *Nitrosospira_sp._9ss1* and *Afipia_sp._1NLS2*

were correlated with the NO_3^- concentration positively ($p < 0.05$). Although some nitrogen conversion microorganisms are involved in the same nitrogen conversion process and have the same gene, their responses to the same environmental variables varied. This may be due to their different sensitivities to different factors or the competitive relationships that exist between them. Under the antibiotics, changes in the nitrogen conversion processes and rates in paddy fields may be related to more detailed changes in different nitrogen conversion bacteria.

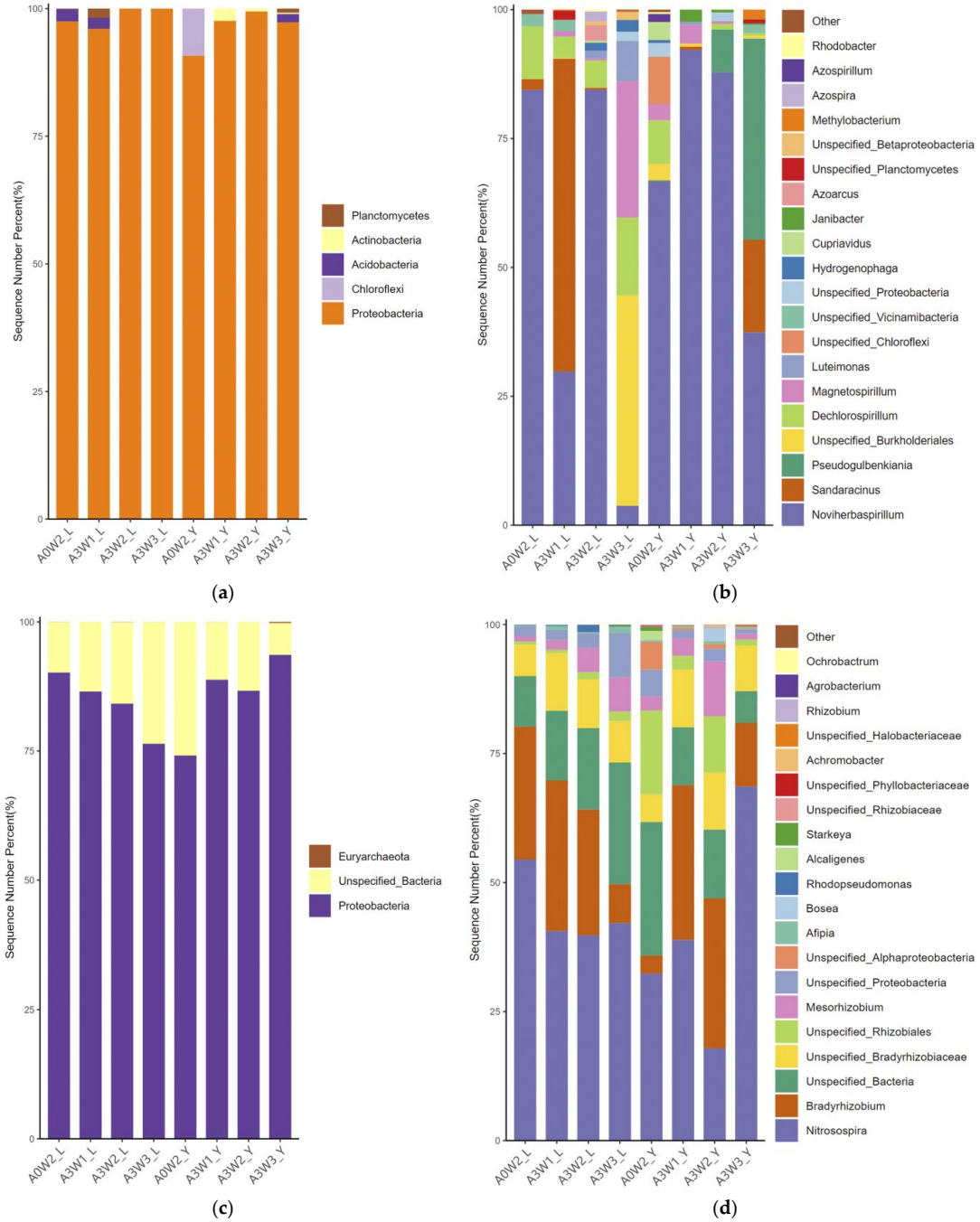
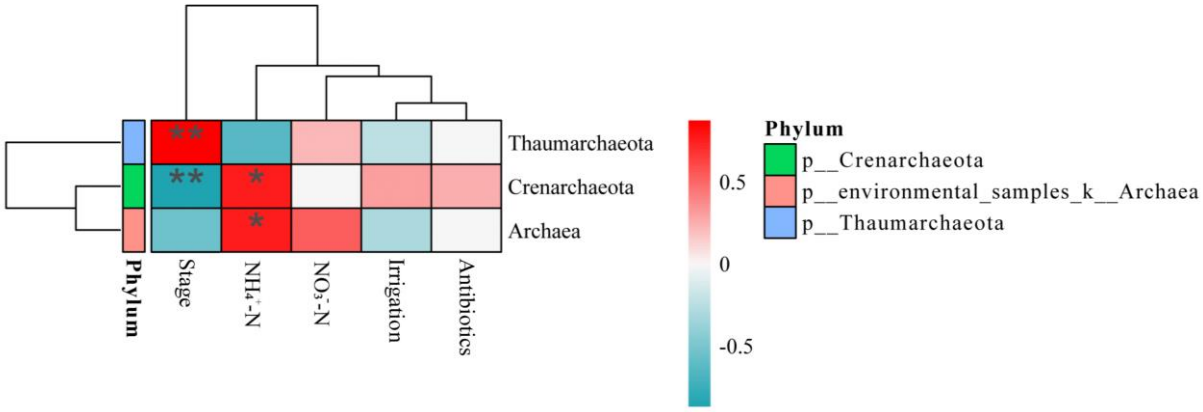
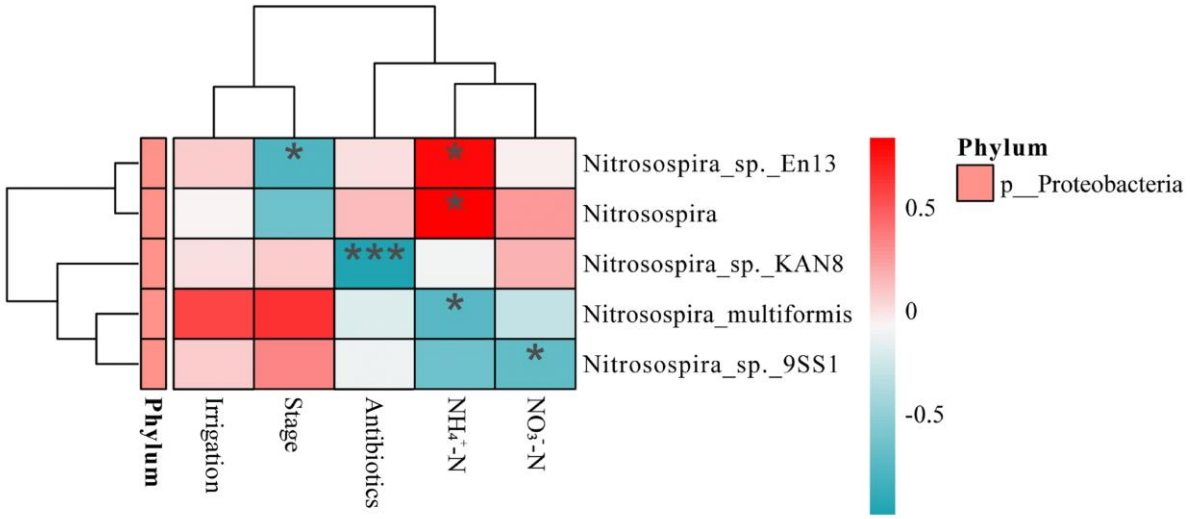


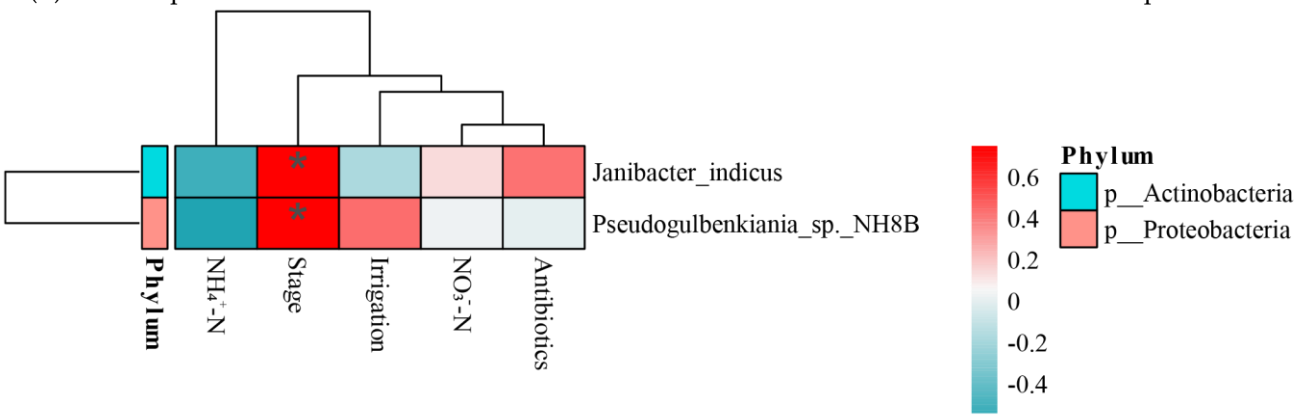
Figure 4. Relative abundance of *nirS* (**a**,**b**) and *nirK*(**c**,**d**) at the phyla and genera levels. A, the concentration gradient of the antibiotic sulfamethazine, including A0 (0 ng·L^{-1}), A1 (500 ng·L^{-1}), A2 (1500 ng·L^{-1}), and A3 (3000 ng·L^{-1}). W, irrigation methods, including W1 (flood irrigation), W2 (mild AWD), and W3 (severe AWD). _L and _Y indicate the late tillering and yellow ripening periods, respectively.



(**a**) Heat map of the correlation between AOA *amoA* archaea and environmental factors at the phyla level

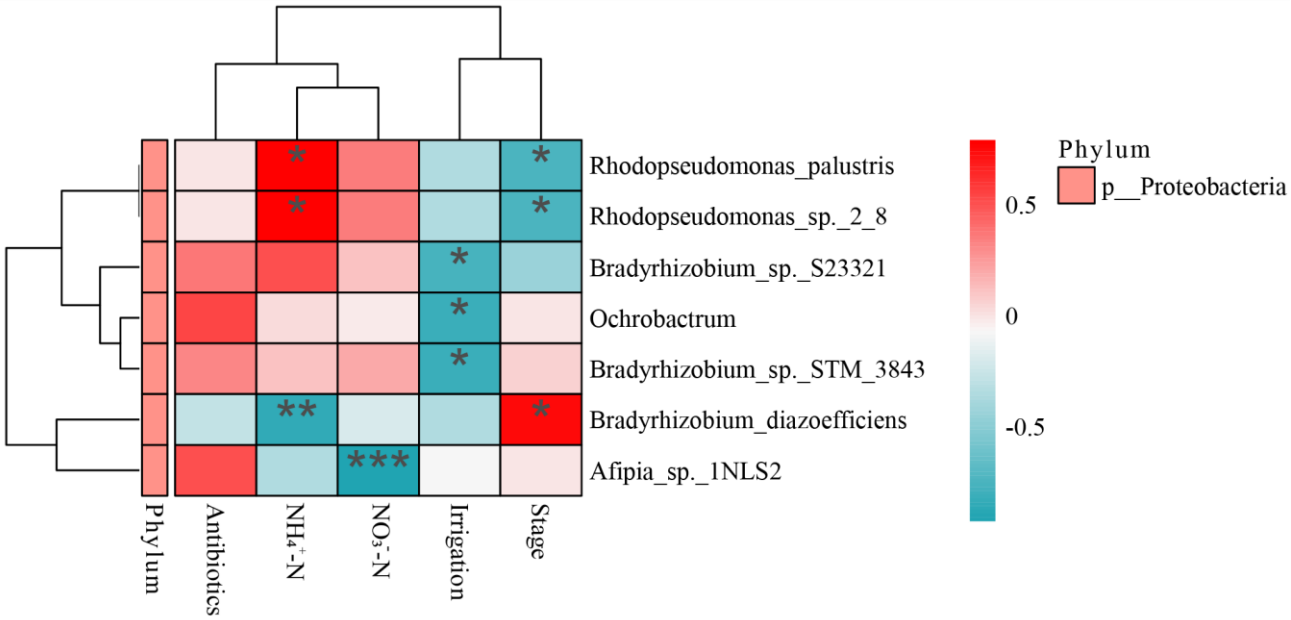


(**b**) Heat map of the correlation between AOB *amoA* bacteria and environmental factors at the species level



(**c**) Heat map of the correlation between *nirS* bacteria and environmental factors at the species level

Figure 5. *Cont.*



(**d**) Heat map of the correlation between *nirK* bacteria and environmental factors at the species level

Figure 5. Heat map of the correlation between nitrogen conversion flora and environmental factors. *, **, and *** indicate that the significance levels of the correlation coefficients are $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Antibiotics, the concentration gradient of the antibiotic sulfamethazine. Irrigation, irrigation methods. NH_4^+-N, the concentration of NH_4^+. NO_3^--N, the concentration of NO_3^-. Stage, growth stages of rice.

4. Discussion

4.1. Soil Nitrogen

The NH_4^+ concentration decreased significantly with the growth stages (Figure 1a). This is roughly the same as the results of Li. [41]. However, the concentration of soil NH_4^+ is also related to the input of exogenous fertilizers, the absorption of nitrogen by rice, the volatile loss of nitrogen ammonia, etc. In this study, there was no significant increase in NH_4^+, and NH_4^+ did not change much in the late tillering and flowering stages of panicle extraction (Figure 1a), which is because the input of fertilizer compensated for the loss of NH_4^+ in the rice growth process. The change of the NO_3^- concentration at different growth stages only showed significant differences at the late jointing and the yellow ripening stages ($p < 0.05$). The regression analysis showed that the regression coefficient is only -0.10 ($p = 0.257$), indicating that NO_3^- did not change significantly in this study. NO_3^- with a negative charge is difficult to be adsorbed by soil colloidal particles but easy to disperse into the environment through leaching [42]. The NO_3^- concentration did not change significantly in this study, and this difference may be because NO_3^- cannot be removed from the pot experiment system. The NO_3^- concentration increased significantly with the increase of the AWD intensity over certain periods, while the $NH4^+$ concentration decreased significantly over certain periods (Figure S2). Cao et al. [43] and Zhang et al. [44] found similar laws to this study in culture experiments. However, the trend of the NO_3^- concentration changing with the AWD intensity gradually changed with the growth period (Figure S2), while the degree of decline of the NH_4^+ concentration with the increase of the intensity of AWD was gradually less obvious with the change of the growth period (Figure S1). The rice demand for nitrogen is mainly concentrated during the reproductive growth period [45]. At the same time, although rice also absorbs NO_3^-, NH_4^+ is still the main source of nitrogen utilization for rice [46]. Therefore, the difference in the responses of NH_4^+ and NO_3^- to AWD may be explained because of the differences in the rice nutrient requirements with growth periods and differences in the rice demand for different types of nitrogen.

Antibiotics can affect the soil nitrogen conversion, but the effects are also related to the type and concentration of antibiotics. Antibiotics affect soil nitrogen conversion by influencing different microorganisms. Revellin et al. [47] found that low concentrations of antibiotics change the diversity and characteristics of nitrogen-fixing bacteria, and many studies have found multiple antibiotics can inhibit nitrification and denitrification [48]. However, some antibiotics, including sulfamethazine, facilitate the denitrification process [27]. Trace antibiotic concentrations were used in this study, but the significant difference of NO_3^- and NH_4^+ still can be observed ($p < 0.05$). Studies have found that low concentrations of antibiotics can promote denitrification, but it only has a significant effect under soil moisture conditions of 40% of the water-filled pore space (WFPS) [49]. The moisture conditions of severe and mild AWD in the study were much greater than 40% WFPS, which may explain the effect of antibiotics in this study is not significant in some cases.

4.2. Nitrogen Conversion-Related Microorganisms

Nitrogen conversion-related microorganisms are important components of the soil microbial community, which includes a variety of microbes involving a variety of nitrogen conversion functional genes. Changes in soil nitrogen conversion-related microorganisms can significantly affect the nitrogen conversion in soil. Many studies have found that, although AOA and AOB participate in the same process of nitrification, their presence in the environment is different. Archaea typically make up 0–10% of the total native microflora in the soil, the most abundant of which is AOA [50]. AOA has been found to occupy higher abundances than AOB in many studies [51–54]. AOA and AOB respond differently to factors such as pH, salinity, NH_4^+, and NO_3^- [55]. AOA is better adapted to low NH_4^+ concentrations, and it has higher abundance at higher NO_3^- concentrations, while AOB tends to have a higher abundance at higher NH_4^+ concentrations [56–58]. In this study, all treatments had higher AOB *amoA* abundances at the late tillering stage, except for A3W2, and A3W1 and A3W3 had higher AOA *amoA* abundances at the yellow ripening stage (Figure 2). Except for A3W2, all treatments had a higher AOA *amoA*/AOB *amoA* ratio at the yellow ripening stage than that at the late tillering stage, which may be due to the high soil NH_4^+ concentration at the late tillering stage. As NH_4^+ was consumed in soils, AOA had a greater advantage in soils. The above discussions show that the main force of nitrification may change as the NH_4^+ and NO_3^- concentrations change in the soil.

The *nirS* and *nirK* genes are important denitrification genes. Abundances of *nirS* are higher in some studies [59]; however, *nirK* have higher abundances in other studies [60–62]. Yoshida et al. [39] found that the abundance of *nirK* was approximately 10 times more than *nirS* throughout the field sampling season. *NirK* is more dominant in this study, and this is due to the fact that *nirS* and *nirK* have different niches. In this study, the Shannon index of AOA *amoA* was between 2.687998 and 3.306445, and the Shannon index of AOB *amoA* was 0.240285–3.391534 (Table 3). *NirK* had the highest Shannon index, because the *nirK* gene had the highest number of species compared to other genes. The community structure of AOA *amoA* changed little and is more stable than AOB *amoA* (Figure 3). At the same time, it was found that AOB *amoA* had a higher correlation coefficient with NH_4^+ and NO_3^- than AOA *amoA*, although they were not significant. However, this may indicate that the intensity change of nitrification in rice fields is mainly contributed to by AOB *amoA*.

Under the impact of antibiotics or moisture conditions, the soil microbial diversity and community structure and the nitrogen cycle processes are changed [63–65]. Further studies show that antibiotics can affect the nitrification and denitrification of soil microorganisms [48,66,67]. This study found that the presence of antibiotics changed the community structure of nitrogen conversion microorganisms, but it also varied at different growth stages of rice (Figures 3 and 4). It is worth noting that, except for AOA *amoA*, A3W2 showed significant differences in the community structures of the other three genes. Although there is a lack of direct evidence that the community structure of AOA *amoA* is more stable than AOB *amoA* under the impact of antibiotics, AOA has been found to be insensitive

to antibiotics [68,69]. The results of this study show that sulfamethazine has a stronger effect on the nitrogen conversion microbial community and nitrogen conversion at a mild AWD. This result may be related to the distribution of antibiotics under such irrigation. Surface tension causes the narrowest diameter pores to control moisture distribution during wetting and drying due to the ink effect. During wetting, the narrow inlet of the large hole slows the infiltration of water, and during the drying process, they obstruct drainage, resulting in water retention, which affects the distribution of nutrients and sulfamethazine in the soil [70]. Therefore, due to the ink effect, the most uniform distribution of nutrients and sulfamethazine may occur under A3W2. Such results suggest that mild AWD may contribute to the effects of antibiotics on soil nitrogen conversion communities and nitrogen conversion processes.

Only *Nitrosospira_sp._KAN8*, belonging to AOB *amoA*, was significantly and negatively correlated with sulfamethazine concentrations (Figure 5a), which may explain why no significant effect of antibiotics on nitrogen conversion has been found in some studies [71,72]. Since the NH_4^+ concentration also changed with the change of the rice growth stages, this study cannot fully distinguish the effects of sulfamethazine on the microbial community structure under long-term action. All the microorganisms involved in this study were nitrifying and denitrifying microorganism. However, they responded to NH_4^+ and NO_3^- differently, and some studies found that AOA *amoA* and AOB *amoA*, *nirS*, and *nirK* respond to environmental factors differently [69,73]. This study further demonstrates that even microbiota with the same nitrogen conversion gene respond significantly differently to environmental factors such as NH_4^+, NO_3^-, and antibiotics. This study may provide a basis for a more in-depth explanation of soil microbial community structures and nitrogen conversion changes.

5. Conclusions

This study found that the presence of antibiotics changes the NH_4^+ and NO_3^- concentrations at the late tillering and jointing–booting stages ($p < 0.05$). Experimental data showed that mild AWD promoted the adverse effect of sulfamethazine (3000 ng/L) on soil nitrogen conversion microorganisms. Microbiota containing the same nitrogen conversion gene exhibited different responses to environmental factors. This study can provide a basis for the use of aquaculture water containing antibiotics to irrigate rice fields. Further research is needed, especially the response of nitrification and denitrification rates to antibiotics and how long antibiotics take to form the effect on nitrogen conversion.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123034/s1: Figure S1: Responses of NH_4^+ concentration to irrigation methods at different growth stages. Figure S2: Responses of NO_3^- concentration to irrigation methods at different growth stages.

Author Contributions: Conceptualization, L.C.; methodology, L.C. and H.W.; formal analysis, L.C., H.W. and W.W.; investigation, W.W. and G.G.; resources, L.C.; data curation, H.W.; writing—original draft preparation, L.C. and H.W.; writing—review and editing, J.L.; visualization, H.W.; project administration, L.C.; and funding acquisition, L.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, (52209052) and the Fundamental Research Funds for the Central Universities (B200204033 and B210202117).

Data Availability Statement: The datasets were deposited in the Sequence Read Archive (SRA) repository under accession# PRJNA891876.

Conflicts of Interest: The authors declare no conflict of interest.

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