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Responses of Alpine Soil Nitrification and Denitrification Rates to Nitrogen Addition Gradient—The Role of Functional Genes

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Abstract: Increases in nitrogen (N) deposition affect the nitrification and denitrification processes that are regulated by microorganisms. With the aim of understanding what happened to the soil nitrification and denitrification rates under the N application gradients, we set a field experiment treated with N at 6 different rates (0, 2, 4, 8, 16, and 32 g $N \cdot m^{-2} \cdot yr^{-1}$) in 2014. We determined the physico-chemical properties, abundances and community structures of the nitrifiers and denitrifiers, the net nitrification rate (NNR) and the potential denitrification rate (PDR) of soil samples that were collected in 2020. We found that the abundances of ammonia-oxidizing bacteria *amoA* (AOB *amoA*), the sum of ammonia-oxidizing archaea *amoA* (AOA *amoA*) and AOB *amoA* increased with the increase in N application rate, but the abundances of *nosZ* decreased with that. The microorganisms that dominate the ammonia oxidation process could shift from AOA to AOB under high N application rates. Furthermore, the soil microorganisms respond to the N addition preferentially with the abundance changes rather than the community composition changes. Moreover, the NNR increased with the N input, while the decrease in the PDR was due to the decrease in the pH value caused by high N application. The results also showed that the *amoA* gene abundance explained most (46.3%) of the variation in the NNR. Moreover, the soil moisture and pH explained 44.0% and 27.1% of the variation in the PDR, respectively. The results demonstrated that the NNR and PDR were mainly explained by functional genes abundances and environmental factors, respectively, in alpine meadow soil under sustained N deposition.

Keywords: nitrogen additions; alpine meadow ecosystem; nitrifier and denitrifier abundances; microbial community structure; nitrification and denitrification rate; soil nitrogen-cycling

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1. Introduction

As nitrogen (N) use in industry and agriculture worldwide has increased continually over recent decades, N deposits from the atmosphere to terrestrial ecosystems have grown rapidly [1]. After a period of rapid socioeconomic transformation, China has implemented programs to monitor and control N pollution. Monitoring data show that N deposition in China began to stabilize around 2000 [2]. However, the current overall tendency toward increases in N deposition worldwide will take some time to change. While N is an essential nutrient for plant and microorganism growth and helps to regulate grassland ecosystem productivity and greenhouse gas emissions [3–5], some ecosystems, including alpine meadows, are sensitive to N deposition.

Numerous researchers have studied how the N additions affect the nitrification and denitrification rates, but the findings to date have not been consistent. For example, a study conducted in grassland found that the net nitrification rate (NNR) increased when N was added [6] and the potential nitrification rate (PDR) did not change noticeably when N was added [7]. Furthermore, meta-analysis showed that the N deposition rate could affect the response of gross N transformations to N deposition [8]. However, meta-analysis also found that the N addition could stimulate the nitrification and denitrification rates [9,10],

and that the response ratio of the nitrification and denitrification rates were positively correlated with N application duration [11]. At present, we have a limited understanding of how the N applications at different rates affect the nitrification and denitrification rates in alpine meadow soils.

Nitrification and denitrification in soil generate N_2O emissions from terrestrial ecosystems that account for about two-thirds of the global N_2O emissions [12]. The rates of soil nitrification and denitrification are regulated by the corresponding functional genes [13]. Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) *amoA* genes generally control the ammonia oxidization process occupying different ecological niches [14,15], so the sum of AOA and AOB *amoA* could reflect the same functional microbial genes abundance, while the AOA:AOB *amoA* ratio could indicate the dominant microbe in the ammonia oxidation process. Nitrite (NO_2^-) reductase is encoded by the *nirK* and *nirS* genes, which control the nitrite (NO_2^-) reduction process, and nitrous oxide (N_2O) reductase, which control N_2O reduction process, is encoded by the *nosZ* gene [13,16,17]. The processes of nitrification and denitrification are controlled by the abundances and community structures of the functional genes [18]. Therefore, it is important to explore the factors that influence the abundances and community structures of the functional genes that drive nitrification and denitrification.

From the meta-analyses, the soil nitrification rate was positively associated with the abundance of the AOB *amoA* gene [11,19]. Researchers also found that the soil nitrification rate had a positive relation with the AOA *amoA* gene abundance [20,21] and that soil pH was the best indicator of the PDR [22]. Moreover, studies indicated that soil moisture was positively correlated to the PDR [23]. It has been shown that environmental factors explain more of the changes in the soil PDR than the abundances of denitrifiers [24]. However, the *nirK* and *nirS* gene abundances were positively correlated with the denitrification rate in some researches [17,25]. The variable results from these studies show that there is little clarity about whether the rates of nitrification and denitrification in soil can be explained by soil functional genes communities or the soil environmental properties.

The overall aim of this scientific research was to probe into how the nitrification and denitrification rates in alpine meadow soil might vary under the N application gradients. To achieve this overall aim, we set up an N application gradient field experiment on the east of the Qinghai–Tibet Plateau to simulate a range of different N deposition patterns. We assumed that the abundances and community structures of nitrifiers and denitrifiers would change because of the N application gradients. We also hypothesized that the nitrification and denitrification rates in the soil would increase when N was added, and that the variations in the soil nitrification and denitrification rates would be influenced mainly by the functional genes abundances.

2. Materials and Methods

2.1. Study Site and Experimental Design

The N application gradient site was located in Sichuan Zoige Alpine Wetland Ecosystem National Observation and Research Station in Hongyuan County, China (32°48′ N, 102°33′ E). The site was 3500 m above sea level. The mean annual air temperature was 1.5 °C, and the mean monthly air temperature was lowest in January (−9.7 °C) and highest in July (11.1 °C). The mean annual precipitation was 758.8 mm (1961–2020), and from May to September with about 80% of rain falling in the alpine meadow. In 2020, the mean monthly air temperature was lowest in January (−7.4 °C) and highest in July (10.9 °C). The annual precipitation was 1033 mm, and from May to September with about 76% of rain falling (Figure S2). Soil (0–20 cm deep) has been classified as Gelic Cambisols in the World Reference Base for Soil Resources. The vegetation cover of the research meadow is higher than 90% and the dominant vegetation species in the region include *Deschampsia caespitosa*, *Kobresia setchwanensi*, *Carex schneideri*, *Anemone rivularis*, and *Oxytropis kansuensis*. The bulk density of the soil (0–20 cm deep) was 0.89 g·cm^{-3}. Total organic carbon and nitrogen contents

of the soil were 37 g C·kg^{-1} and 3.5 g N·kg^{-1}, respectively [26]. The background N deposition on the eastern Qinghai–Tibetan Plateau was reported as 1.23 ± 0.28 g N·m^{-2}·yr^{-1} [27].

The experimental area for the N applications was established in early 2014. It contains 30 plots (8 × 8 m) with a 3-m buffer between adjacent plots. The experiment was designed as a randomized complete block and the plots receive the N applications with N at 6 different rates (0, 2, 4, 8, 16, and 32 g N·m^{-2}·yr^{-1}, described here as N0, N2, N4, N8, N16, N32, respectively) and each treatment has 5 replicates. The plots are arranged randomly in the research area (Figure S1). N is applied manually on the surface every month from May to September (the growing season) before rainfall as dry NH_4NO_3.

2.2. Soil Sampling and Physico-Chemical Analysis

In August 2020, soil samples from surface to 20 cm were collected by a core sampler (D = 5 cm) before N was added in August. Three cores of soil were randomly gathered from each plot and were sieved through a 2-mm mesh. Then, one part of the soil samples was stored at −20 °C for analyzing physico-chemical properties. The other part was stored at −80 °C for DNA extraction. The soil moisture (SM) was determined after drying 10 g fresh soil in an oven at 65 °C to constant weight. The pH meter was used to test pH value (SevenCompact, Mettler Toledo, Switzerland). The soil leaching liquor was extracted with 0.5 mol·L^{-1} K_2SO_4 (40 mL). Inorganic N contents (NH_4^+-N, NO_3^--N) were analyzed by a continuous flow analyzer (AA3, Bran Luebbe, Hamburg, Germany). TOC element analyzer (Liquid TOCII, Elementar, Frankfurt, Germany) was used to determine dissolved organic carbon (DOC) content. The methods for measuring the soil properties have been described elsewhere in detail [28].

The NNR of the soil was determined by the incubator culture method [29]. Fresh soil (10 g) was placed in a 250 mL triangle beaker and then the soils were cultured in an incubator at 25 °C for 14 days. The soil leaching liquor was extracted with 2 mol·L^{-1} KCl (50 mL) before and after culturing. The NO_3^--N content of the extracted solution was measured with a gas phase molecular absorption spectrometer (AJ−3000 Plus, China). The change in the NO_3^--N content per gram of dry soil before and after culturing was used to calculate the NNR of the soil in mg NO_3^--N·g^{-1} soil·d^{-1}.

The PDR was determined by the acetylene gas inhibition method [30]. The soil samples (weight of dry soil converted to 4 g) and ultra-pure water (the total water volume was 13 mL) were placed into a 100-mL anaerobic bottle. The anaerobic bottle was shaken at room temperature for 20 min. Then the anaerobic bottle was pumped to vacuum and filled with 10% (*V/V*) acetylene (an N mixture was used to balance the air pressure). The acetylene gas was mixed by vibrating at room temperature for 20 min. DEA solution (20 mL), prepared by dissolving 56 mg KNO_3-N, 288 mg glucose-C, and 2 mg chloramphenicol in 1 L sterile water, was added to the anaerobic bottle. A sample of gas (20 mL) was extracted before culturing, and another sample of gas (20 mL) was extracted after culturing at room temperature for 2 h. The N_2O contents were determined by gas chromatograph (Aglilent 7890A GC System, Santa Clara, CA, USA). The difference between the N_2O contents before and after culturing represented the PDR of the soil, measured in ng N_2O-N h^{-1}·g^{-1} dry soil.

2.3. Quantitative PCR and High-Throughput Sequencing

The total soil DNA was extracted by a Power Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) within two months since the soil was collected. The concentration of the extracted DNA was determined with a spectrophotometer (Nanodrop Technologies, USA), and then we calculated the microbial DNA concentration per dry soil (ug·g^{-1}) and defined this index as the microbial biomass (MB). The abundances of AOA *amoA*, AOB *amoA*, *nirS*, *nirK*, and *nosZ* were quantified by 7500 Fast Real-Time PCR (Applied Biosystems). The 20 µL PCR reaction system and gene primer pairs and amplification conditions are shown in Table S1 and Table S2. The amplification efficiency and R^2 values were controlled at 80–110% and 0.995–0.999.

The functional genes were sequenced on a high-throughput sequencing platform (Illumina Miseq PE300) with a measured flux of 60,000 pieces. The amplification primers and the functional gene amplification conditions are shown in Table S3. The sequencing data were inspected and filtered through the existing codes of the Meige Gene cloud platform (http://cloud.magigene.com/login, accessed on 1 July 2021), and then were classified into operational taxonomic units (OTU) with a 98% similarity. The raw reads of five genes were submitted into the NCBI Sequence Read Archive under accession numbers PRJNA878936, PRJNA878640, PRJNA878634, PRJNA876623, PRJNA876569.

2.4. Data Analysis

The differences between all the N application gradients were determined by one-way ANOVA with a significance level of 0.05 (SPSSv20.0). Data were first tested for normality and transformed. The LSD test was used to compare the groups for indicators of homogeneity of variance (NO_3^--N, DOC, AOA *amoA*, AOA *amoA* + AOB *amoA*, and AOA *amoA*:AOB *amoA*). The Dunnet-T3 test was used to compare between groups for indicators of heterogeneity of variance (SM, NH_4^+, MB, AOB *amoA*, *nirK*, *nirK* + *nirS*, NNR, and PDR). Data that could not be normally transformed (pH, *nirS*, *nosZ*, and *nirK:nirS*) were analyzed with the Kruskal–Wallis test. The correlations between the soil properties and the functional gene abundances were determined using the Spearman correlation test (SPSS v 20.0). The bacterial community and soil physico-chemical property data were subjected to redundancy analysis (RDA) in Canoco 5. The factors driving the nitrification and denitrification rates were explored using stepwise regression (SPSSv20.0). The differences in the compositions of the functional gene communities were tested using principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity and non-parametric multiple variance analysis (Adonis test) using Rv4.2.0. The figures were drawn using Origin2018.

3. Results

3.1. The Effects of N Application with Different Rates on Soil Physico-Chemical Properties

Summary information about the properties of the soil treated with the different N applications is provided in Figure 1. The pH was lower in the N16 and N32 treatments than in N0 (Figure 1b). The NO_3^- contents were increased under the high N application rates (N8, N16, and N32 treatments) compared to N0 (Figure 1d). The N16 and N32 treatments facilitated the DOC contents compared to N0 (Figure 1e), and the MB was lower in the N8, N16, and N32 treatments than in N0 (Figure 1f). Further, the SM and the NH_4^+ contents were similar for all the N application gradients (Figure 1a,c).

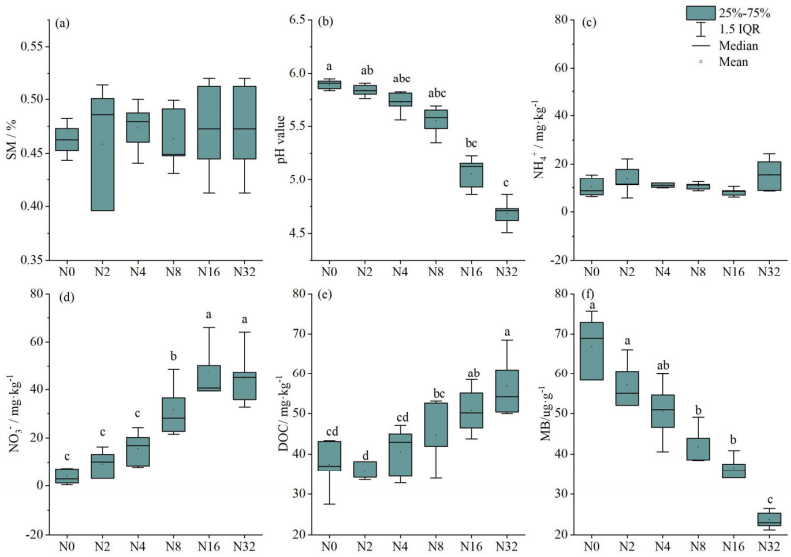

Figure 1. Boxplots showing the physico-chemical properties of the soils with different N application

rates. (**a**) Soil moisture (SM), (**b**) pH, (**c**) NH_4^+ contents, (**d**) NO_3^- contents, (**e**) DOC contents, and (**f**) microbial biomass (MB). In all, 25–75% represents the interquartile range (IQR). The different lowercase letters indicate significant differences among all the N application treatments ($p < 0.05$). N0 is the control (0 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application), N2 is the 2 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N4 is the 4 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N8 is the 8 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N16 is the 16 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, and N32 is the 32 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application.

3.2. The Effect of Different N Application Rates on the Abundances of Nitrifiers and Denitrifiers

The five gene abundances responded differently to the N applications (Figure 2). The AOA *amoA* gene abundance was similar in all the N application gradients (Figure 2a). The AOB *amoA* gene abundances were facilitated in the high N application rates (N8, N16, and N32) compared to N0 (Figure 2b, $p < 0.05$). The *amoA* gene abundances (the sum of AOA and AOB *amoA*) were higher in all the N application gradients than in N0 (Figure 2c, $p < 0.05$). We found that AOA:AOB *amoA* ratio > 1 under N0 treatments, while AOA:AOB *amoA* ratio < 1 under N2 to N32 treatments. The AOA:AOB *amoA* ratio was significantly higher in the control than in the N2, N8, N16, and N32 treatments (Figure 2d, $p < 0.05$). Additionally, the AOA:AOB *amoA* ratio was lower for the high N application rates (N16, and N32) than the low N application rates (N2 and N4) applications. The *nirK*, *nirS*, and *nir* gene abundances (the sum of *nirK* and *nirS*) were similar for all 6 N application gradients (Figure 2e,f); however, the *nosZ* gene abundance was lower for the N32 treatment than for the N0 (Figure 2i). The *nirK*:*nirS* ratio ranged from 1.12 to 1.19 and did not vary significantly between the N treatments (Figure 2h).

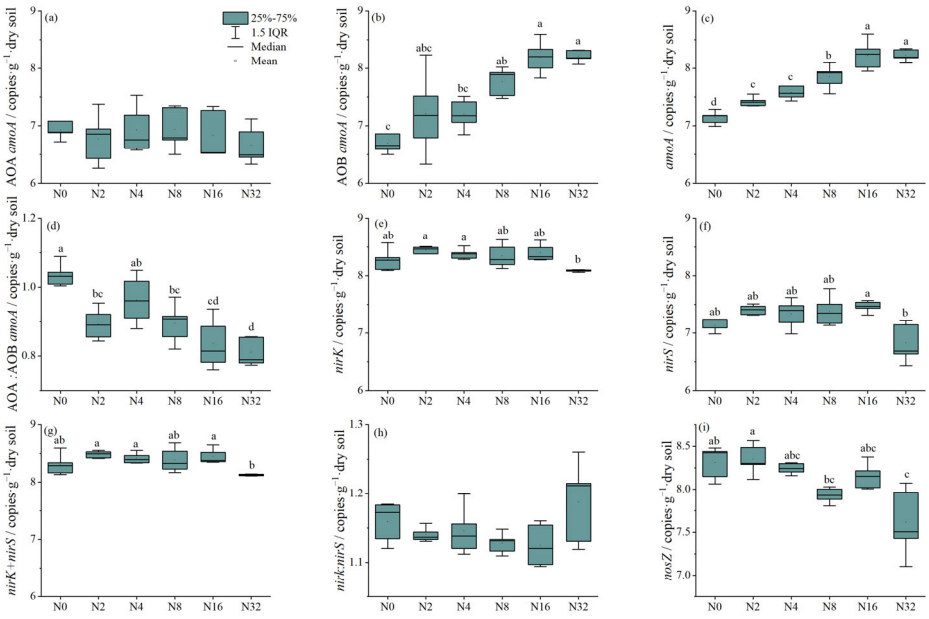

Figure 2. Boxplot of the abundances of the functional genes of the soil nitrifiers and denitrifiers under different N application rates. (**a**) gene abundance of AOA *amoA*, (**b**) gene abundance of AOB *amoA*, (**c**) gene abundance of *amoA*, (**d**) the ratio of AOA:AOB *amoA*, (**e**) gene abundance of *nirK*, (**f**) gene abundance of *nirS*, (**g**) gene abundance of (*nirK* + *nirS*), (**h**) the *nirK*:*nirS* ratio, and (**i**) nosZ. In all, 25–75% represents the interquartile range (IQR). Different lowercase letters indicate significant differences among all the N application treatments ($p < 0.05$). N0 is the control (0 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application), N2 is the 2 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N4 is the 4 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N8 is the 8 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, N16 is the 16 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application, and N32 is the 32 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ application.

3.3. The Effects of the Different N Addition Rates on the Compositions of the Nitrifier and Denitrifier Communities

We identified the differences in the community structures in the soils treated with the five levels of N and the control using principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity. The Adonis test was used to highlight significant differences between the pairs of the different N treatments. The results showed that the community structures between the pairs of the N treatments were not significantly different (Table S4).

The AOA *amoA* functional group was mainly comprised of *Nitrososphaerales*, *Nitrosopumilales*, and *Nitrosocaldales* (Figure 3a). The *Nitrosospira* in the AOB *amoA* functional group were mainly from *Cluster-1*, *Cluster-3b*, and *Cluster-8b* (Figure 3b). The *nirK* and *nirS* functional groups were mainly comprised of *α-*, *β-*, and *γ-proteobacteria* (Figure 4a,b). The *nosZ* functional groups were mainly comprised of *Rhizobiales*, *Pseudomonadales*, *Rhodospirillales*, *Burkholderiales*, *Rhodobacterales*, *Campylobacterales*, and *Alteromonadales* (Figure 4c).

3.4. Relationships between the Soil Physical and Chemical Properties and the Abundances and Community Structure of Nitrifier and Denitrifier

The AOB *amoA* and *amoA* gene abundances had a negative relation with the pH, but were positively correlated with the N application rate, NO_3^- contents, and DOC contents ($p < 0.05$, Table 1). The *nirK*, *nirS*, and *nir* gene abundances had a positive relation with the soil SM ($p < 0.05$, Table 1). The *nosZ* gene abundance and MB had a positive relation with the pH but had a negative relation with the N application rate, NO_3^- contents, and DOC contents ($p < 0.05$, Table 1).

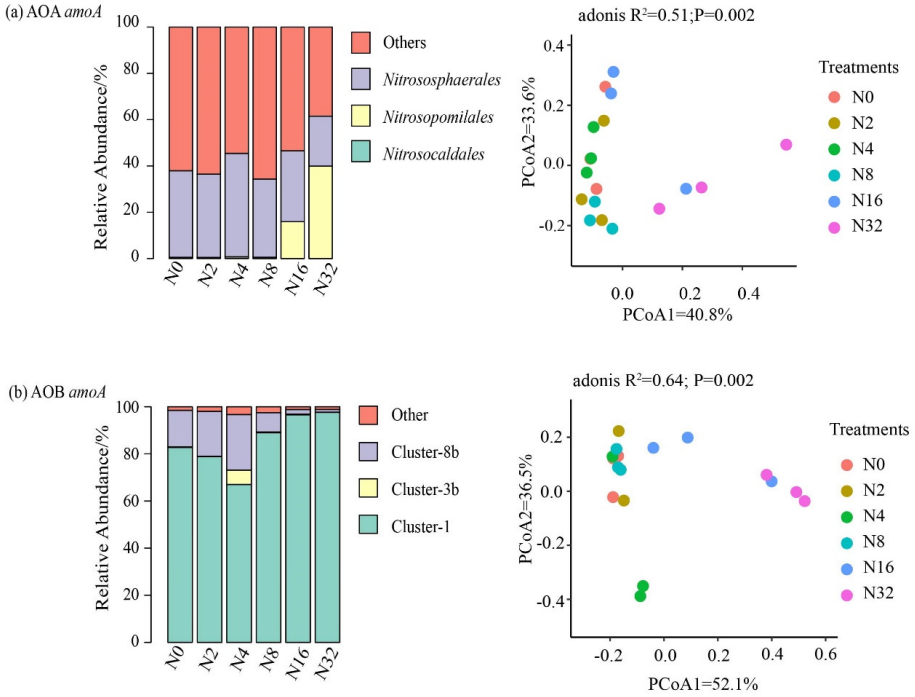

Figure 3. The relative abundances of nitrifier community compositions and principal coordinates analysis (PCoA) based on the Bray–Curtis distance. (**a**) AOA *amoA*, and (**b**) AOB *amoA*. N0 is the control (0 g $N \cdot m^{-2} \cdot yr^{-1}$ application), N2 is the 2 g $N \cdot m^{-2} \cdot yr^{-1}$ application, N4 is the 4 g $N \cdot m^{-2} \cdot yr^{-1}$ application, N8 is the 8 g $N \cdot m^{-2} \cdot yr^{-1}$ application, N16 is the 16 g $N \cdot m^{-2} \cdot yr^{-1}$ application, and N32 is the 32 g $N \cdot m^{-2} \cdot yr^{-1}$ application.

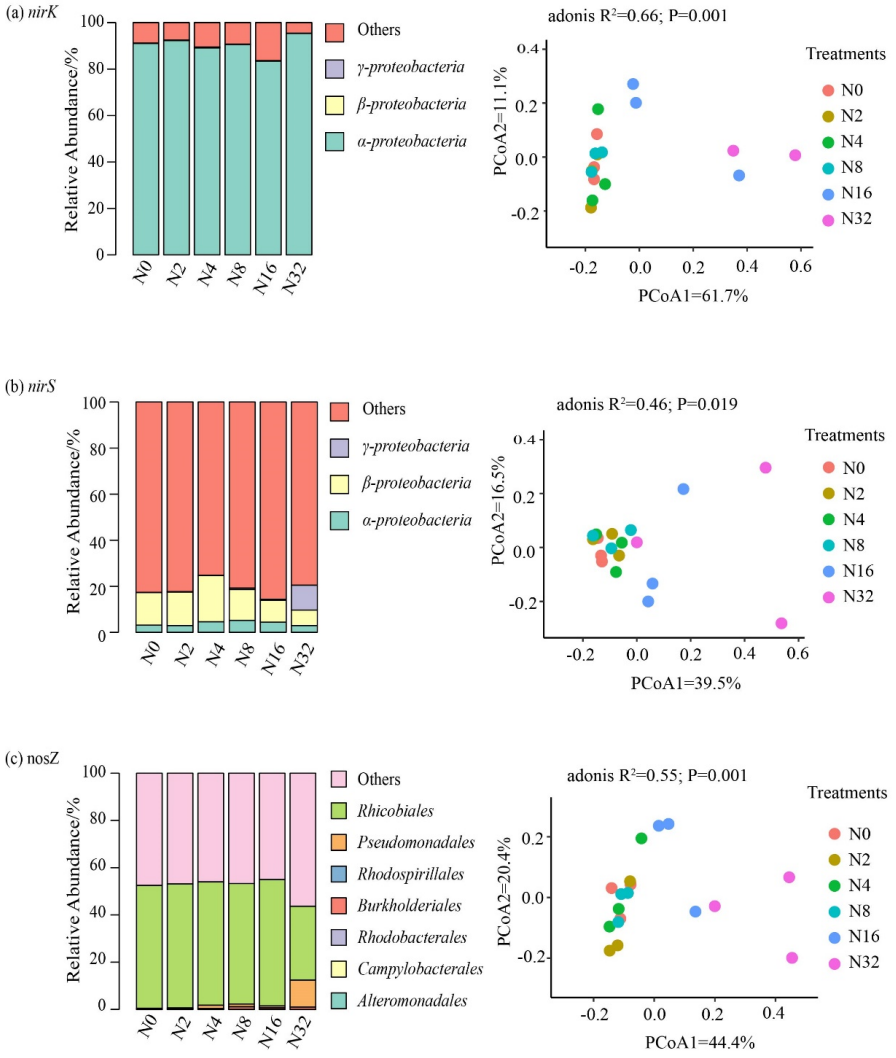

Figure 4. The relative abundances of nitrifier community compositions and principal coordinates analysis (PCoA) based on the Bray–Curtis distance. (**a**) *nirK*, (**b**) *nirS*, (**c**) *nosZ*. N0 is the control (0 g N·m^{-2}·yr^{-1} application), N2 is the 2 g N·m^{-2}·yr^{-1} application, N4 is the 4 g N·m^{-2}·yr^{-1} application, N8 is the 8 g N·m^{-2}·yr^{-1} application, N16 is the 16 g N·m^{-2}·yr^{-1} application, and N32 is the 32 g N·m^{-2}·yr^{-1} application.

Table 1. Spearman's correlation coefficients for the relationships between the nitrifier and denitrifier abundances and the soil physical and chemical properties.

	AOA	**AOB**	***amoA***	***nirK***	***nirS***	***nir***	***nosZ***	**MB**
N	−0.185	0.819 **	0.941 **	−0.333	−0.05	−0.316	−0.671 **	−0.940 **
SM	−0.006	0.217	−0.035	0.433 *	0.530 **	0.467 **	0.368 *	0.328
pH	0.161	−0.846 **	−0.922 **	0.304	0.034	0.295	0.582 **	0.876 **
NH_4^+	0.214	0.07	0.015	0.013	−0.085	−0.037	−0.15	0.113
NO_3^-	−0.1	0.788 **	0.902 **	−0.126	0.205	−0.098	−0.555 **	−0.789 **
DOC	−0.136	0.696 **	0.729 **	−0.236	−0.175	−0.221	−0.486 **	−0.684 **

* Significant at the 0.05 probability level. ** Significant at the 0.01 probability level. N is the nitrogen application rate. SM is the soil moisture. DOC is dissolved organic carbon. MB is microbial biomass. *amoA* is (AOA *amoA* + AOB *amoA*). *nir* is (*nirK* + *nirS*).

The results from the RDA to explore the main influencing factors of the nitrifier and denitrifier bacterial communities are shown in Figure 5. The pH (F = 18.1, p = 0.002), NO_3^- (F = 3.4, p = 0.048), and DOC (F = 3.6, p = 0.030) were the main environmental influences on

the AOA *amoA* bacterial communities (Figure 5a, $p < 0.05$) and explained 68.5% of the total variation. The NO_3^- contents (F = 6.7, $p = 0.014$) and the soil moisture (F = 8.7, $p = 0.010$) were the main environmental influences on the AOB *amoA* bacterial communities (Figure 5b, $p < 0.05$) and explained 45.4% of the total variation. The pH (F = 5.7, $p = 0.002$) was the main influence on the *nirS* bacterial communities (Figure 5d, $p < 0.05$) and explained 36.3% of the total variation. The DOC contents (F = 5.9, $p = 0.008$), NH_4^+ contents (F = 5.4, $p = 0.006$), and the soil moisture (F = 4.6, $p = 0.028$) were the main environmental influences on the *nosZ* bacterial communities (Figure 5e, $p < 0.05$) and explained 45.3% of the total variation. However, the *nirK* bacterial communities were not influenced by the environmental factors (Figure 5c).

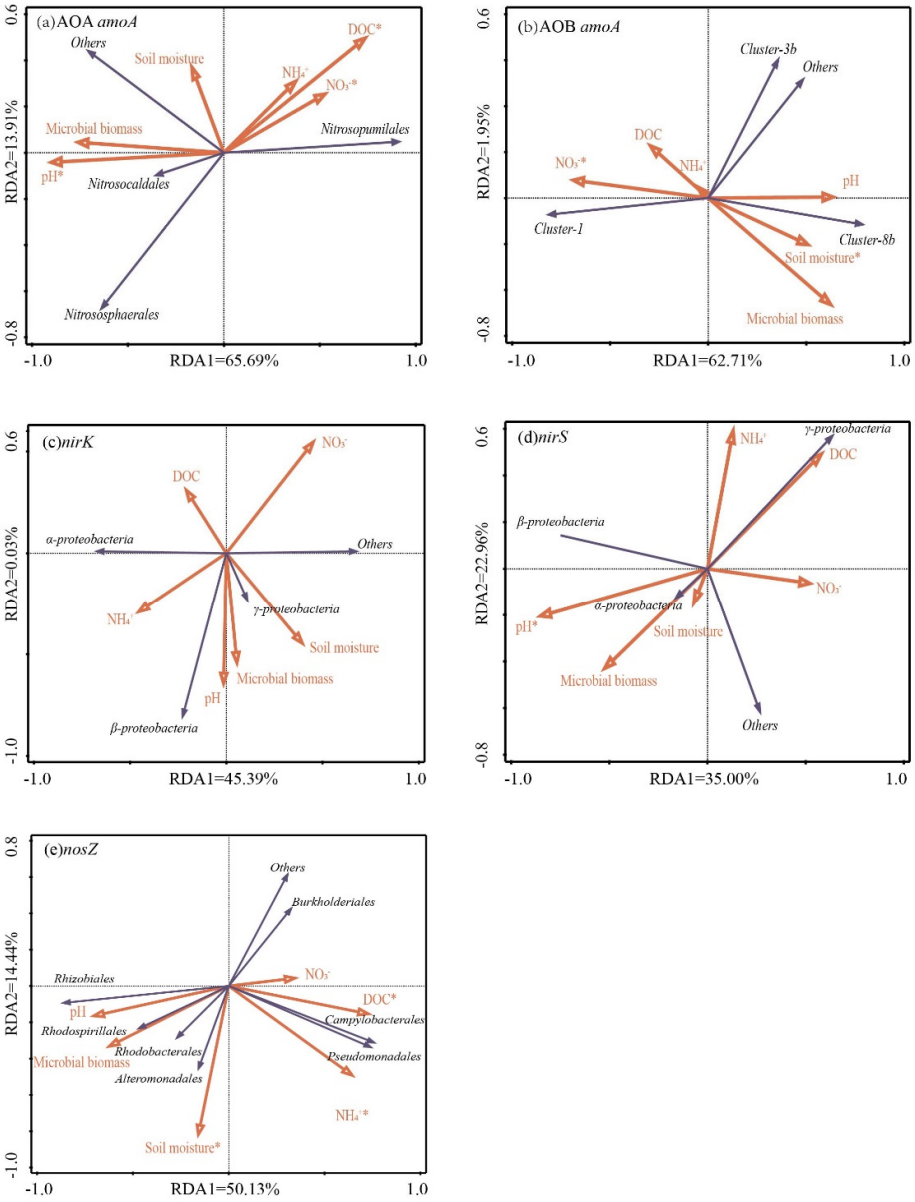

Figure 5. Redundancy analysis (RDA) showing the correlations between nitrifiers (AOA *amoA* (**a**), AOB *amoA* (**b**)) and denitrifiers (*nirK* (**c**), *nirS* (**d**) and *nosZ* (**e**)) bacterial communities and the soil physico-chemical properties. * Significant at the 0.05 probability level. SM is the soil moisture. DOC is dissolved organic carbon. MB is microbial biomass.

3.5. Soil Nitrification and Denitrification Rates in the Soils Treated with Different N Applications

The NNR ranged from 17.38 to 224.29 ug NO_3^--N·d^{-1}·g^{-1} soil. The NNR was higher for all the N application rates than for the control (N0) (Figure 6a, $p < 0.05$) and was highest for the N16 treatment. The PDR ranged from 112.62 to 577.99 ng N_2O-N·h^{-1}·g^{-1} dry soil. When compared with the control, the different N applications had no significant effect on the PDR (Figure 6b, $p < 0.05$), and only the PDR in the N32 treatment was lower than that in the control (29.37% lower).

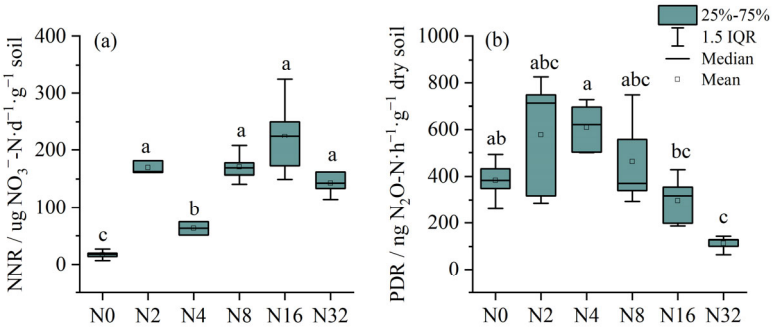

Figure 6. Boxplot of the soil nitrification and denitrification rates under different N application rates. (**a**) Net nitrification rate (NNR), and (**b**) potential denitrification rate (PDR). In all, 25−75% represents the interquartile range (IQR). The different lowercase letters indicate significant differences among all the different N application treatments ($p < 0.05$). N0 is the control (0 g N·m^{-2}·yr^{-1} application), N2 is the 2 g N·m^{-2}·yr^{-1} application, N4 is the 4 g N·m^{-2}·yr^{-1} application, N8 is the 8 g N·m^{-2}·yr^{-1} application, N16 is the 16 g N·m^{-2}·yr^{-1}application, and N32 is the 32 g N·m^{-2}·yr^{-1}application.

The results from stepwise multiple regression to show the main environmental and microbial influences on nitrification and denitrification are presented in Table 2. The gene abundance of *amoA* explained most (46.3%) of the variation in the NNR (Table 2, $p < 0.001$), while the soil moisture and pH explained most (44.0% and 27.1%, respectively) of the variations in the PDR (Table 2, $p < 0.001$).

Table 2. Results of multiple regression analyses applied to data obtained from all N treatments to evaluate the relative importance of microbiological and environmental factors for the net nitrification activities (NNA) and potential denitrification activities (PDA).

	Factor (n = 30)	**Standardized Beta**	***p***	**ss**
NNR	*amoA* abundance	0.694	<0.001	0.463
	Total explained			0.463
PDR	SM	0.555	<0.001	0.44
	pH	0.535	<0.001	0.271
	Total explained			0.711

4. Discussion

4.1. Impacts of N Applications on the Abundances of Soil Nitrifiers and Denitrifiers

In our research, the N application could promote the net nitrification rate, which produces H^+ and decreases the soil pH value (Fig1, b). Meanwhile, N could increase the abundances of nitrifiers (the sum of AOA and AOB *amoA*), and the pH value had a negative relationship with the abundance of AOB *amoA* and the sum of AOA and AOB *amoA* (Table 1), which indicated that the N addition could improve the ability of soil to convert ammonium to nitrate and caused the result that the soil ammonium concentration was not increased with the N inputs. Furthermore, soil acidification caused by high N application could decrease the *nosZ* abundance (Figure 2, $p < 0.05$), demonstrating that the microorganisms were not always limited by N and that high N supplements might

negatively impact the soil microbial abundance [31], which in turn may decrease the reduction in N_2O to N_2 and increase the greenhouse gas N_2O emission.

Meanwhile, we found that the response of the AOB *amoA* gene abundance to the N application was more susceptive than the AOA *amoA* gene. All the N addition gradients had no effect on the AOA *amoA* gene abundance, but the high N application rates increased the AOB *amoA* gene abundance compared to the N0 treatment. These results are consistent with an earlier study of an alpine meadow [32]. The different responses of AOA and AOB may reflect their different physiological characteristics, metabolic patterns, and habitat preferences, as they are located in different ecological niches [33]. AOA has a smaller volume and a larger specific surface area than AOB, and the ammonia monooxygenase (AMO) of AOA has a stronger affinity for ammonia nitrogen than that of AOB, so the AMO of AOA can reach saturation at a lower NH_4^+ concentration, meaning that AOA does not change significantly when N is applied [14]. Meanwhile, we found that the AOA:AOB *amoA* ratio was > 1 under the N0 treatments, which means that AOA dominates the soil ammonia-oxidizing process, while the AOA:AOB *amoA* ratio was < 1 under the N2 to N32 treatments, which means that AOB dominates the soil ammonia-oxidizing process. Additionally, the AOA:AOB *amoA* ratio was lower for the high N application rates (N8, N16, and N32) compared to the low N application rate (N2 and N4) applications, which suggests that the microorganisms that dominate the ammonia oxidation process could shift from AOA to AOB under the N application rates.

Comparison with the control (N0) showed that the N applications had no effect on *nirK*, *nirS*, and *nir* (Figure 2). This finding is consistent with a meta-analysis by Dong et al. [34], who found that denitrifying functional genes (*nirK* and *nirS*) in grassland were stable under the N additions, which suggests that the functional gene abundances of the denitrifying microorganisms were not sensitive to the N additions in alpine meadows. The *nirK*:*nirS* ratio ranged from 1.12 to 1.19 under the N treatments and did not differ for the different N additions, which displays that *nirK* dominated the denitrification process numerically and the relative dominance of *nirK* and *nirS* did not change under the N applications.

We found that the AOB *amoA* gene abundance had a positive relation with the NO_3^- contents (Table 1, $p < 0.01$), but was not related to the NH_4^+ contents (Table 1). That was due to the fact that the N inputs could significantly promote the nitrification rate but not the denitrification rate in our research. The nitrification process could convert ammonium into nitrate and caused the result that the soil nitrate but not the ammonium concentration had a positive relationship with N application rates. Furthermore, the *nirK*, *nirS*, and *nosZ* abundances had a positive relation with the SM (Table 1, $p < 0.01$), as denitrification prefers anaerobic conditions [13]; when the SM is high, soil porosity could decrease and anaerobic conditions could develop.

4.2. Impacts of N Applications on the Nitrifier and Denitrifier Community Compositions

The nitrifier and denitrifier community compositions in these soils were not affected by any N application gradients, indicating that the nitrifying and denitrifying groups in this alpine meadow soil were stable and resistant to changes in nutrient conditions. Meanwhile, considering that the N addition could significantly change gene abundances of nitrifiers and denitrifiers, the results suggest that the soil microorganisms respond to the N addition preferentially with the abundance changes rather than the community composition changes. Other studies have reported similar findings. For example, the community compositions of the AOA *amoA*, AOB *amoA*, *nirK,* and *nirS* functional groups in both temperate and subtropical forests did not change after N was applied for 3 years (10 g $N \cdot m^{-2} \cdot yr^{-1}$) [25]. However, after N was applied to temperate steppe soils for 8 years, the community compositions of the AOB *amoA* and *nosZ* functional groups changed significantly, but the community compositions of the AOA *amoA*, *nirK,* and *nirS* functional groups did not change [7]. Moreover, another study found that the AOB *amoA* group shifted, and the AOA *amoA* group did not shift, when N was added over a period of 3 years (10 g $N \cdot m^{-2} \cdot yr^{-1}$) [32]. These studies show that the N application duration and rate may

be important for shaping the nitrifying and denitrifying groups. Moreover, the responses of the nitrifying and denitrifying groups to the N applications may vary by ecosystem type.

The RDA results highlighted that the pH was the main environmental influence on the AOA *amoA* bacterial communities (Figure 5a, $p < 0.05$), which is consistent with the results of previous research [35]. In acidic conditions, NH_3 might be transformed to NH_4^+, and the bioavailability of the NH_3 might affect how the different AOA *amoA* communities adapt [36]. This study showed that NO_3^- was the main environmental influence on the AOB *amoA* bacterial communities (Figure 5b, $p < 0.05$); other studies, however, reported that NH_4^+ was the strongest influence on the structure of the AOB *amoA* group [35]. This difference may be evidence that ammonium was converted into NO_3^- after the nitrification reaction, as NO_3^- had a strong influence on the community structure of the AOB *amoA* group. The *nirS* bacterial communities were strongly influenced by the pH (Figure 5d, $p < 0.05$), but the *nirK* bacterial communities were not influenced by the environmental factors (Figure 5c). This shows that the two functional groups had different sensitivities to the soil properties [37,38]. The DOC, NH_4^+, and the soil moisture were the main environmental influences on the *nosZ* bacterial communities (Figure 5e, $p < 0.05$), which suggests that the N additions might affect the community structure of the *nosZ* functional group through changing the microbial energy and substrate.

4.3. Impacts of N Applications on Nitrification and Denitrification Rates

The N additions triggered different changes in the NNR and PDR. The NNR was higher for all the N application rates than for the control (N0) (Figure 6a, $p < 0.05$). The increase in the nitrification rate may reflect the weaker competition of plants rather than the microorganisms for N when the inorganic N substrate is added [6]. The PDR for the N32 treatment was 29.37% less than that of the N0 treatment (Figure 6b, $p < 0.05$), which shows that the soil denitrification processes remained stable when low N was applied. Research also found that the N applications had no effect on the PDR in forest and grassland soil [25,39]. The decrease in the PDR under the N32 treatment may reflect the complexity of the PDR process in soil, from their meta-analysis, concluded that the soil PDR was affected by the N contents, MB, pH, water content, texture, and temperature of the soil [40]. That the PDR of the N32 soil was lower than in the other soils may reflect the acidic conditions.

From their meta-analysis, the AOB *amoA* abundance had a positive correlation with the potential nitrification rate in numerous ecosystem types [19]. Conversely, studies found that the nitrification rate was related to the AOA *amoA* abundance [21], while research showed that AOA and AOB both contribute to ammonia oxidation [20]. Here, we found that the sum of the AOA *amoA* and AOB *amoA* gene abundances explained most of the NNR (Table 2, $p < 0.001$), which suggests that the functional gene abundance was the most important influence on the soil nitrification rate. Moreover, we found that the PDR was strongly influenced by the soil moisture and pH (Table 2, $p < 0.001$). These results are coincident with a range of factors. The soil pH was the best indicator of the PDR [22]. The denitrification rate was also affected by the soil moisture, through influencing the fluidity and aeration of the soil solutes. When the soil moisture is high, the nitrification rate decreases and the denitrification rate increases [23]. In this study, the nitrifier abundances were more sensitive to the N applications than the denitrifier abundances. The nitrification rate was mainly explained by the nitrifier abundances, while the denitrification rate was mainly explained by environmental factors. The results may indicate that the abundance of the denitrifying functional genes does not respond significantly to the N addition, and that environmental factors may be an important explanation for the denitrification rate. The results were different with a meta-analysis that showed that the *narG* gene abundance was an important control factor of the denitrification rate under the N deposition [11], which may reflect that the environmental factor may be an important explanation for the denitrification rate when the abundance of the denitrifying functional genes does not respond significantly to the N addition.

5. Conclusions

Our results show that the nitrifiers and NNR were more sensitive to N fertilization than the denitrifiers and PDR in alpine meadows, which indicates that soil acidification was mainly affected by the nitrification process producing H^+. The N application could change the microorganisms that dominate the ammonia-oxidizing process from AOA to AOB. High N application decreased the *nosZ* gene abundance, which may increase N_2O emission to the atmosphere. Moreover, the community compositions of AOA *amoA*, AOB *amoA*, *nirS*, *nirK*, and *nosZ* were not affected by the N addition, which indicates that the soil microorganisms respond to the N addition preferentially with the abundance changes rather than the community composition changes. Our study suggests that the nitrification rate was mainly explained by the nitrifier abundance (46.3%), while the denitrification rate was mainly explained by soil environmental properties (71.1%). These findings will provide a theoretical basis for future model simulation of the nitrification and denitrification processes and suggest that functional gene abundances and soil environmental properties need to be considered more in the simulation of nitrification and denitrification, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020245/s1. Figure S1: Nitrogen application gradient experimental design; Figure S2: Mean monthly temperature and monthly precipitation of the filed site in 2020; Table S1: PCR reaction system; Table S2: Primers and amplification conditions of PCR for functional gene abundances; Table S3: Primers and amplification conditions of PCR for functional gene species composition; Table S4: Primers and amplification conditions of PCR for functional gene species composition.

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References

1. Galloway, J.N.; Townsend, A.R.; Erisman, J.W.; Bekunda, M.; Cai, Z.; Freney, J.R.; Martinelli, L.A.; Seitzinger, S.P.; Sutton, M.A. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science* **2008**, *320*, 889–892. [CrossRef] [PubMed]
2. Yu, G.; Jia, Y.; He, N.; Zhu, J.; Chen, Z.; Wang, Q.; Piao, S.; Liu, X.; He, H.; Guo, X.; et al. Stabilization of atmospheric nitrogen deposition in China over the past decade. *Nat. Geosci.* **2019**, *12*, 424–429. [CrossRef]
3. Luo, Q.; Gong, J.; Yang, L.; Li, X.; Pan, Y.; Liu, M.; Zhai, Z.; Baoyin, T.-T. Impacts of nitrogen addition on the carbon balance in a temperate semiarid grassland ecosystem. *Biol. Fertil. Soils* **2017**, *53*, 911–927. [CrossRef]
4. Xu, C.; Xu, X.; Ju, C.; Chen, H.Y.H.; Wilsey, B.J.; Luo, Y.; Fan, W. Long-term, amplified responses of soil organic carbon to nitrogen addition worldwide. *Glob. Chang. Biol.* **2021**, *27*, 1170–1180. [CrossRef]
5. Zhang, J.; He, P.; Liu, Y.; Du, W.; Jing, H.; Nie, C. Soil properties and microbial abundance explain variations in N_2O fluxes from temperate steppe soil treated with nitrogen and water in Inner Mongolia, China. *Appl. Soil Ecol.* **2021**, *165*, 103984. [CrossRef]
6. Ma, L.N.; Lu, X.T.; Liu, Y.; Guo, J.X.; Zhang, N.Y.; Yang, J.Q.; Wang, R.Z. The effects of warming and nitrogen addition on soil nitrogen cycling in a temperate grassland, northeastern China. *PLoS ONE* **2011**, *6*, e27645. [CrossRef]
7. Zhang, C.J.; Yang, Z.L.; Shen, J.P.; Sun, Y.F.; Wang, J.T.; Han, H.Y.; Wan, S.Q.; Zhang, L.M.; He, J.Z. Impacts of long-term nitrogen addition, watering and mowing on ammonia oxidizers, denitrifiers and plant communities in a temperate steppe. *Appl. Soil Ecol.* **2018**, *130*, 241–250. [CrossRef]
8. Song, L.; Li, Z.; Niu, S. Global Soil Gross Nitrogen Transformation Under Increasing Nitrogen Deposition. *Glob. Biogeochem. Cycle* **2021**, *35*, e2020GB006711. [CrossRef]

9. Lu, M.; Yang, Y.; Luo, Y.; Fang, C.; Zhou, X.; Chen, J.; Yang, X.; Li, B. Responses of ecosystem nitrogen cycle to nitrogen addition: A meta-analysis. *New Phytol.* **2011**, *189*, 1040–1050. [CrossRef]
10. Wang, J.; Chadwick, D.R.; Cheng, Y.; Yan, X. Global analysis of agricultural soil denitrification in response to fertilizer nitrogen. *Sci. Total Environ.* **2018**, *616*, 908–917. [CrossRef]
11. Song, L.; Niu, S. Increased soil microbial AOB amoA and narG abundances sustain long-term positive responses of nitrification and denitrification to N deposition. *Soil Biol. Biochem.* **2022**, *166*, 108539. [CrossRef]
12. Thomson, A.J.; Giannopoulos, G.; Pretty, J.; Baggs, E.M.; Richardson, D.J. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philos. Trans. R. Soc. B Biol. Sci.* **2012**, *367*, 1157–1168. [CrossRef]
13. Canfield, D.E.; Glazer, A.N.; Falkowski, P.G. The Evolution and Future of Earth's Nitrogen Cycle. *Science* **2010**, *330*, 192–196. [CrossRef]
14. Prosser, J.I.; Nicol, G.W. Archaeal and bacterial ammonia-oxidisers in soil: The quest for niche specialisation and differentiation. *Trends Microbiol.* **2012**, *20*, 523–531. [CrossRef]
15. Purkhold, U.; Pommereningröser, A.; Juretschko, S.; Schmid, M.C.; Koops, H.P.; Wagner, M. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Appl. Environ. Microbiol.* **2000**, *66*, 5368–5382. [CrossRef]
16. Shiina, Y.; Itakura, M.; Choi, H.; Saeki, Y.; Hayatsu, M.; Minamisawa, K. Relationship Between Soil Type and N_2O Reductase Genotype (*nosZ*) of Indigenous Soybean Bradyrhizobia: *nosZ*-minus Populations are Dominant in Andosols. *Microbes Environ.* **2014**, *29*, 420–426. [CrossRef]
17. Yin, C.; Fan, F.; Song, A.; Li, Z.; Yu, W.; Liang, Y. Different denitrification potential of aquic brown soil in Northeast China under inorganic and organic fertilization accompanied by distinct changes of nirS- and nirK-denitrifying bacterial community. *Eur. J. Soil Biol.* **2014**, *65*, 47–56. [CrossRef]
18. Ullah, S.; Ai, C.; Huang, S.H.; Song, D.L.; Abbas, T.; Zhang, J.J.; Zhou, W.; He, P. Substituting ecological intensification of agriculture for conventional agricultural practices increased yield and decreased nitrogen losses in North China. *Appl. Soil Ecol.* **2020**, *147*, 103395. [CrossRef]
19. Carey, C.J.; Dove, N.C.; Beman, J.M.; Hart, S.C.; Aronson, E.L. Meta-analysis reveals ammonia-oxidizing bacteria respond more strongly to nitrogen addition than ammonia-oxidizing archaea. *Soil Biol. Biochem.* **2016**, *99*, 158–166. [CrossRef]
20. He, J.; Shen, J.; Zhang, L.; Zhu, Y.G.; Zheng, Y.; Xu, M.; Di, H.J. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environ. Microbiol.* **2007**, *9*, 2364–2374. [CrossRef]
21. Isobe, K.; Ikutani, J.; Fang, Y.; Yoh, M.; Mo, J.; Suwa, Y.; Yoshida, M.; Senoo, K.; Otsuka, S.; Koba, K. Highly abundant acidophilic ammonia-oxidizing archaea causes high rates of nitrification and nitrate leaching in nitrogen-saturated forest soils. *Soil Biol. Biochem.* **2018**, *122*, 220–227. [CrossRef]
22. Sun, P.; Zhuge, Y.; Zhang, J.; Cai, Z. Soil pH was the main controlling factor of the denitrification rates and N-2/N_2O emission ratios in forest and grassland soils along the Northeast China Transect (NECT). *Soil Sci. Plant Nutr.* **2012**, *58*, 517–525. [CrossRef]
23. Cheng, Y.; Wang, J.; Wang, S.-Q.; Zhang, J.-B.; Cai, Z.-C. Effects of soil moisture on gross N transformations and N_2O emission in acid subtropical forest soils. *Biol. Fertil. Soils* **2014**, *50*, 1099–1108. [CrossRef]
24. Shrewsbury, L.H.; Smith, J.L.; Huggins, D.R.; Carpenter-Boggs, L.; Reardon, C.L. Denitrifier abundance has a greater influence on denitrification rates at larger landscape scales but is a lesser driver than environmental variables. *Soil Biol. Biochem.* **2016**, *103*, 221–231. [CrossRef]
25. Tang, Y.G.; Yu, G.R.; Zhang, X.Y.; Wang, Q.F.; Tian, D.S.; Tian, J.; Niu, S.L.; Ge, J.P. Environmental variables better explain changes in potential nitrification and denitrification activities than microbial properties in fertilized forest soils. *Sci. Total Environ.* **2019**, *647*, 653–662. [CrossRef] [PubMed]
26. Song, B.; Sun, J.; Zhou, Q.; Zong, N.; Li, L.; Niu, S. Initial shifts in nitrogen impact on ecosystem carbon fluxes in an alpine meadow: Patterns and causes. *Biogeosciences* **2017**, *14*, 3947–3956. [CrossRef]
27. Wen, Z.; Xu, W.; Li, Q.; Han, M.; Tang, A.; Zhang, Y.; Luo, X.; Shen, J.; Wang, W.; Li, K.; et al. Changes of nitrogen deposition in China from 1980 to 2018. *Environ. Int.* **2020**, *144*, 106022. [CrossRef]
28. Liu, Y.; Zhang, J.; Li, Y.; He, P.; Dong, J. Do long-term N additions affect the soil organic carbon pool in temperate grasslands? *Sci. Total Environ.* **2021**, *810*, 152227. [CrossRef]
29. Wang, C.; Wan, S.; Xing, X.; Zhang, L.; Han, X. Temperature and soil moisture interactively affected soil net N mineralization in temperate grassland in Northern China. *Soil Biol. Biochem.* **2006**, *38*, 1101–1110. [CrossRef]
30. Smith, M.S.; Tiedje, J.M. Phases of denitrification following oxygen depletion in soil. *Soil Biol. Biochem.* **1979**, *11*, 261–267. [CrossRef]
31. Treseder, K.K. Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. *Ecol. Lett.* **2008**, *11*, 1111–1120. [CrossRef]
32. Xiang, X.; He, D.; He, J.-S.; Myrold, D.D.; Chu, H. Ammonia-oxidizing bacteria rather than archaea respond to short-term urea amendment in an alpine grassland. *Soil Biol. Biochem.* **2017**, *107*, 218–225. [CrossRef]
33. Offre, P.; Kerou, M.; Spang, A.; Schleper, C. Variability of the transporter gene complement in ammonia-oxidizing archaea. *Trends Microbiol.* **2014**, *22*, 665–675. [CrossRef]

34. Dong, J.Y.; Zhang, J.Q.; Liu, Y.H.; Jing, H.C. How climate and soil properties affect the abundances of nitrogen-cycling genes in nitrogen-treated ecosystems: A meta-analysis. *Plant Soil* **2022**, *477*, 389–404. [CrossRef]
35. Chen, Z.; Guo, Y.; Du, Z.; Wu, W.; Meng, F. Change in the abundance and community composition of ammonia-oxidizing bacteria and archaea at soil aggregate level as native pasture converted to cropland in a semiarid alpine steppe of central Asia. *J. Soils Sediments* **2015**, *16*, 243–254. [CrossRef]
36. Nicol, G.W.; Leininger, S.; Schleper, C.; Prosser, J.I. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ. Microbiol.* **2008**, *10*, 2966–2978. [CrossRef]
37. Yang, Y.D.; Hu, Y.G.; Wang, Z.M.; Zeng, Z.H. Variations of the nirS-, nirK-, and nosZ-denitrifying bacterial communities in a northern Chinese soil as affected by different long-term irrigation regimes. *Environ. Sci. Pollut. Res. Int.* **2018**, *25*, 14057–14067. [CrossRef]
38. Yang, Y.; Nie, J.; Wang, S.; Shi, L.; Li, Z.; Zeng, Z.; Zang, H. Differentiated responses of nirS- and nirK-type denitrifiers to 30 years of combined inorganic and organic fertilization in a paddy soil. *Arch. Agron. Soil Sci.* **2020**, *67*, 79–92. [CrossRef]
39. vanGestel, M.; Merckx, R.; Vlassak, K. Spatial distribution of microbial biomass in microaggregates of a silty-loam soil and the relation with the resistance of microorganisms to soil drying. *Soil Biol. Biochem.* **1996**, *28*, 503–510. [CrossRef]
40. Zhaolei, L.; Tang, Z.; Song, Z.; Chen, W.; Tian, D.; Tang, S.; Wang, X.; Wang, J.; Liu, W.; Wang, Y.; et al. Variations and controlling factors of soil denitrification rate. *Glob. Chang. Biol.* **2021**, *28*, 2133–2145. [CrossRef]