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Nitrogen Significantly Affected N Cycling Functional Gene Abundances Compared with Phosphorus and Drought in an Alpine Meadow

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Abstract: Human activities and global climate change have greatly increased nitrogen (N) and phosphorus (P) inputs and altered precipitation patterns in alpine meadows. Functional genes are important indicators of microorganisms that drive the nitrogen cycling process; however, the functional gene responses of soil nitrogen cycling to soil N and P availability and drought remain unclear. Separate or combined treatments of nitrogen and phosphorus fertilization and drought were conducted on the Zoige Wetland in the Qinghai-Tibet Plateau, and the abundances of nitrification functional genes AOA *amoA* and AOB *amoA* and denitrification functional genes *nirS*, *nirK*, and *nosZ* were measured to explore the response of functional genes to these treatments. Seven treatments, including control (CK), N addition (N), P addition (P), 50% reduction in precipitation (D), N and P addition (NP), N addition with drought (ND), and NP addition with drought (NPD), were investigated. The results indicated that N application significantly increased AOB *amoA* abundance, while P application and drought had no significant effects on the abundance of functional genes. The combined treatment of N and P addition and drought increased AOB *amoA* abundance but did not significantly affect AOA *amoA* abundance, suggesting that AOB *amoA* was more responsive to soil N and P availability and moisture change than AOA *amoA*. However, the abundance of denitrification functional genes was not affected by these treatments. Denitrification functional genes were less sensitive to soil N and P availability and moisture change than nitrification functional genes. The integrated effects of N addition, P addition, and drought did not affect the abundance of the above N cycling functional genes. These results indicate that AOB *amoA* may play a more critical role in the process of ammonia oxidation than AOA *amoA* in alpine meadows, and the denitrification genes (*nirK*, *nirS*, and *nosZ*) were better than ammoxidation genes (AOA and AOB) at adapting to the soil environmental changes caused by increasing N and P deposition and drought in alpine meadows.

Keywords: differential response; drought; interaction effect; N cycling functional gene; nitrogen addition; phosphorus addition

Citation: Li, H.; Zhang, J.; Tian, D.; Liu, Y.; Dong, J. Nitrogen Significantly Affected N Cycling Functional Gene Abundances Compared with Phosphorus and Drought in an Alpine Meadow. *Agronomy* **2023**, *13*, 1041. https://doi.org/10.3390/agronomy13041041

Academic Editor: Lili Jiang

Received: 30 January 2023
Revised: 13 March 2023
Accepted: 29 March 2023
Published: 31 March 2023

1. Introduction

The response of nitrification and denitrification genes that regulate the N cycle to changes in nitrogen, phosphorus, and precipitation patterns in soil has received extensive attention [1–3]. Although many studies have revealed the influence of N and P nutrient increases and precipitation changes on N cycling functional genes, most of them are limited to the response of functional genes to single or two resources, such as N and P addition [4], N and precipitation [5], and P and precipitation [6]. However, there are few studies on the interaction and mechanism of various soil resource changes on soil N cycling functional

genes [7,8]. Many studies have shown that precipitation affects functional gene abundances by changing the availability of oxygen in soil [9], but the mechanism by which N and P nutrient application impact microorganisms in the case of precipitation decrease remains to be studied.

Nitrification and denitrification processes complete the N cycle in the soil. Ammoxidation is the first step of nitrification and is catalyzed by two functional microbial communities: ammoxidation archaea (AOA *amoA*) and ammoxidation bacteria (AOB *amoA*). The reduction of NO_2^- to NO is the first step in the denitrification process and is catalyzed by two different nitrite reductases encoded by the *nirK* or *nirS* gene. NO is further converted to N_2O, and N_2 is produced under the catalysis of nitrous oxide reductase encoded by *nosZ*. Most N addition experiments in alpine meadows changed the abundance of AOB *amoA*, while AOA *amoA* abundance remained constant, indicating that AOB *amoA* was more responsive to N fertilization than AOA *amoA* [10]. AOB *amoA* is an active nitrifying bacterium and plays a more critical role in the ammonia oxidation process [11]. Most studies have shown that ammonia oxidation in acidic soils is dominated by AOA *amoA* [12], and recent research has also indicated that AOB *amoA* is dominant [13]. N application alone did not affect functional gene abundance, while P application significantly changed the abundance of most N cycling functional genes, and the promoting effect of P was offset by N when P was applied together with N [4]. AOA *amoA* was more active and abundant than AOB *amoA* when P and N were applied in the alpine meadow [14]. The abundance of *nirS*, *nirK*, and *nosZ* may be decreased [15], increased [16], or insensitive [11,17] to N and P fertilization, which are not easily affected in alpine meadows [11]. Decreased precipitation may reduce microbial richness [18] and inhibit N_2O emissions, which may mean a positive impact on *nosZ* abundance [19], and N deposition may exacerbate the effects of drought [20]. Compared with nitrification, denitrification is rarely studied because it is difficult to quantify the final products of denitrification [17]. The response difference of N cycling functional genes to changes in N, P, and precipitation needs to be further studied.

The application of N significantly affected functional gene abundance by changing soil properties, mainly ammonia substrate concentration and pH [21]. P addition may accelerate N mineralization and thus promote ammonia oxidation [6]. Precipitation affects the abundance of functional genes by changing the soil microbial activities [22] and species diversity [23], nutrient limitation [24], and soil microclimate [25]. The increase in precipitation can alleviate the negative impact of N fertilizer on soil pH reduction [26], and the input of N counteracts the promoting effect of P by reducing the availability of P [4]. Song et al. noted that pH and soil-dissolved organic carbon (DOC) determine the role of AOA and AOB in ammoxidation [27]. AOA *amoA* was thought to have a better affinity for ammonia substrates, to be more adaptable to acidic environments, and to dominate in moist soils [28], whereas, under long-term fertilization, AOB *amoA* appeared to be more resistant to acidification and to occupy a broader ecological niche [7]. Denitrification functional genes were more affected by water content because of anaerobic conditions. The abundance of denitrification functional genes decreased with increasing water and N [22]. Watering increased the abundance of *nirS* in temperate semiarid grassland [29]. A meta-analysis showed that the impact of precipitation change on *nirS* and *nosZ* abundances was U-shaped with soil moisture [18]. Studies have shown that the abundance of denitrification functional genes is significantly correlated with soil organic carbon (SOC) and pH [30]. In addition, ecosystem type [31] and the form [32], and duration [33] of fertilization are also important reasons for the difference in response to nutrient and environmental changes between ammonia-oxidizing bacteria and archaea. However, the joint mechanism of the response to N, P, and precipitation reduction remains unclear. Therefore, we studied the effects of N, P, and precipitation changes on functional genes of the N cycle and their interaction effects to understand the future N cycle under environmental changes.

Since the mid-20th century, the Tibetan Plateau has experienced unprecedented human disturbances, including grazing and fertilization [34], and the P and N sedimentation of the Qinghai-Tibet Plateau affected by human factors has continued to increase [34]. With

the increase in human activities on the Qinghai-Tibet Plateau, many studies have paid attention to the influence of N and P fertilization on N cycling functional genes. Most studies showed that AOB *amoA* abundance was changed by nitrogen application, while other cycling functional genes were not affected [10]. Although the effects of N and P fertilization on soil N cycling genes on the Tibetan Plateau have been investigated [14], few experiments have been conducted to explore the interaction between P and N application and precipitation reduction. The response and sensitivity of denitrification functional genes to environmental changes in alpine meadows remain to be explored.

In this study, the interaction of N and P addition and drought on N cycling genes and their interaction in alpine meadows were examined. The abundances of AOA *amoA*, AOB *amoA*, *nirS*, *nirK,* and *nosZ* were measured. In addition, the soil's physical-chemical characteristics, soil temperature (ST), and soil water content (SWC) were determined. The objectives of the study were to investigate (1) the response and difference in N cycling functional gene abundances to changes in multiple resources and (2) the response mechanism of N cycling functional gene abundances to multiple resource changes and the reasons for the differences in response.

2. Materials and Methods

2.1. Experimental Area and Experimental Design

The research was carried out in the Sichuan Zoige Alpine Wetland Ecosystem National Observation and Research Station (32°49′59″ N, 102°34′53″ E, and 3490 m above sea level) in Hongyuan County, Aba Tibetan and Qiang Autonomous Prefecture, Sichuan Province. The study area has a continental plateau cold temperate semihumid monsoon climate. According to the record of the Hongyuan climatological station, the average annual temperature at the experimental site is 1.4 °C, and the average annual precipitation is 650–750 mm. The area is dominated by alpine meadow and swamp soil and is covered with alpine meadow vegetation [35,36]. The soil water content is 34.74%, and the soil pH is 5.96.

The experimental design was completed in May 2017 and was included in two global standard network trials of the Nutrient Network and Drought Network in 2018. A randomized block experimental design was adopted, which included 7 treatments: control (CK), N fertilization (N), P fertilization (P), drought (D), N and P combined fertilization (NP), N fertilization and drought (ND), and NP fertilization and drought (NPD), in which drought refers to a 50% precipitation reduction. A sketch map of the location of research sample plots and the layout of experimental plots have been shown by Fu et al. [37]. Each treatment was repeated 4 times for a total of 28 plots measuring 4 m × 4 m, with a distance of 2 m between each plot. The addition of nitrogen and phosphorus was 10 g/(m^2·a), the nitrogen fertilizer was resin-coated urea (N content is 46.6%), and the phosphorus fertilizer was calcium superphosphate (P_2O_5 content is 12.0%). The fertilization concentration and drought treatment were consistent with those of the Nutrient Network [38] and Drought Network [39]. At the beginning of May every year, continuous rainy days were selected to evenly spread nitrogen and phosphorus fertilizers in the corresponding treatment plots. Resin transparent PVC board (light transmittance: 99%) was built on the steel frame structure greenhouses with a length and width of 6 m and a height of 3 m for 50% precipitation reduction (D) treatment.

2.2. Soil Sampling and Physico-Chemical Analysis

On a rain-free day in the middle of August 2020, a soil sampler with a diameter of 3 cm was used to gather soil samples at a depth of 20 cm. Each plot was sampled 3 times at random and then mixed thoroughly. Then, the mixed soil was sifted through a 2 mm mesh and packed into 2 plastic self-sealing bags. One of the samples was prepared to analyze physical and chemical properties and stored at −20 °C, and the other was used for DNA extraction and soil microbial assays and stored at −80 °C.

Ten grams of each sample was soaked in 40 mL 0.5 M K_2SO_4 and then filtered [29]. The NO_3^--N and NH_4^+-N contents (mg/kg) of the filtered liquid were measured with a flow analyzer (AA3, Bran Luebbe, Germany), and the DON content (mg/kg) was measured with a TOC analyzer (TOC-L CPN, Shimadzu, Kyoto, Japan,) [40]. The soil samples were air-dried and ground, sifted with a 0.25 mm sieve, and the total nitrogen (TN) content (mg/g) was obtained with an elemental analyzer (Vario MAX CN, Elementar, Hanau, Germany). The soil was pickled with 10% HCl to determine the content of total organic nitrogen (TON, mg/g). Ten grams of each sample was fumigated with chloroform for approximately 24 h and then soaked in 40 mL 0.5 M K_2SO_4 and then filtered [41]. The extract was analyzed with a TOC analyzer (TOC-L CPN, Shimadzu, Kyoto, Japan). The determination result divided by the coefficient 0.54 is the microbial biomass nitrogen (MBN, mg/kg) content. After the air-dried soil (10 g) and distilled water (40 mL) were mixed uniformly and then left to settle, the pH of the suspension was measured with a pH analyzer (PB-10). Soil temperature (ST, °C) was measured at a depth of 0–10 cm using a Li-6400 portable temperature probe, and soil water content (SWC) was measured at a depth of 0–10 cm using a TDR hydrometer (TDR350, Aurora, IL, USA). The ST and SWC used in this analysis are the average values of the annual growing season measured twice a month from May to September 2020.

2.3. DNA Extraction and Real-Time PCR

The sample was stored at −80 °C and then thawed, and 0.5 g of each sample was weighed. A FastDNA TM SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) was used to extract the total DNA of soil according to the instructions. A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) was used to measure the concentration and purity of the obtained DNA. The abundances of 5 N cycle genes *nirK*, *nirS*, *nosZ*, AOA *amoA*, and AOB *amoA* were determined with a real-time PCR instrument (7500 Fast Real-Time PCR, Thermo Fisher Scientific, Waltham, MA, USA). The plasmid synthesis and PCR procedure were described by Tang et al. [32,41] and the 10-fold continuously diluted plasmids of each gene were used in qPCR to generate a standard curve. The DNA extracts were tested for inhibitory effects of coextracted substances by series dilution before quantification [42]. The DNA negative controls, sample replication, and standard series were performed in 96 well plates. A total of 0.2 μL of the forward primer, 0.2 μL of the reverse primer (20 mM), 10 μL of TaKaRa Premix (TaKaRa), 1 μL of the DNA sample and 8.6 μL of ddH_2O constituted the 20 μL qPCR mixture. The R^2 values were greater than 0.995, and the PCR efficiency was between 85% and 110%. The gene primers and the temperature and time of the qPCR reaction have been described by Zhang et al. [29], and more information about standard curves have been explained by Tang et al. [41].

2.4. Data Analysis

Before analysis, the abundances of genes were log-transformed to satisfy the homogeneity of variance assumption. One-way ANOVA was used to analyze the effects of N addition, P addition, and drought on soil physical and chemical properties and gene abundance. Three-way ANOVA was used to analyze the interactions of N addition, P addition, and drought on gene abundance. Pearson correlation analysis and a mixed linear model were applied to search for the main factors explaining the abundance of functional genes. Variance analysis and Pearson correlation analysis were conducted with IBM SPSS Statistics27 (SPSS, Armonk, New York, NY, USA), and the mixed linear model was generated with the lmerTest and lme4 packages in R Studio. The figures were generated using Origin2018.

3. Results

3.1. Effects of Different Treatments on the Abundances of Functional Genes

N and P addition and drought treatment exerted modest effects on the functional gene abundances, significantly affecting the abundance of AOB *amoA* and AOA + AOB. One-way ANOVA results showed that the AOB *amoA* abundance in NPD-treated soil

was significantly higher than that in D-treated and CK soil, and the total abundance of AOA + AOB in N-treated soil was greater than that in D-treated and CK soil ($p < 0.05$, Figure 1). Moreover, the AOA *amoA* abundance in D-treated soil was significantly less than that in N-treated soil, but the abundance of AOA *amoA* was not significantly different between different treatments and CK. The total abundance of AOA *amoA* and AOB *amoA* in NPD-treated soil was significantly greater than that in D-treated soil ($p < 0.05$, Figure 1). However, there was no significant difference in the ratio of AOA *amoA* abundance to AOB *amoA* abundance among the different treatments (Figure 1), so the relative abundance of AOA *amoA* and AOB *amoA* may not be affected by N treatment, P treatment, D treatment, or their combinations. Denitrification functional gene abundances were insensitive to these treatments, as different treatments had no significant effects on the copy numbers of *nirS*, *nirK*, and *nosZ* (Figure 2). The abundance of *nirK* + *nirS* and the ratio of the *nirK* to *nirS* gene abundance did not change significantly under these different treatments (Figure 2). Three-way ANOVA suggested that N addition had a significant influence on the abundance of AOB *amoA* and AOA *amoA* + AOB *amoA* ($p < 0.05$, Table 1). Other experimental treatments did not significantly affect the abundance of functional genes, and the interaction of N, P, and D treatments was not significant (Table 1).

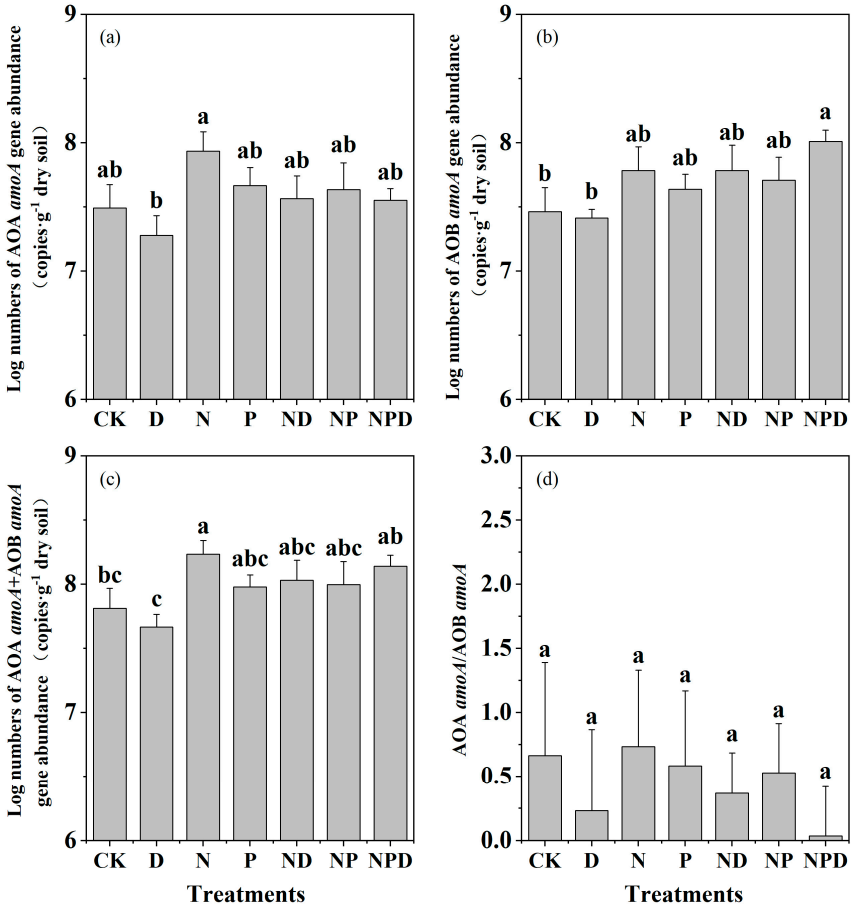

Figure 1. Effects of different treatments on the abundances of the (**a**) AOA *amoA* gene; (**b**) AOB *amoA* gene; (**c**) AOA *amoA* gene + AOB *amoA* gene; and (**d**) AOA *amoA* / AOB *amoA*. Note: Data are presented as the mean ± SE (n = 4). Different letters above boxplots indicate significant differences ($p < 0.05$) between treatments. Treatments: control (CK), drought (D), nitrogen addition (N), phosphorus addition (P), nitrogen and drought combined (ND), nitrogen and phosphorus combined (NP), and nitrogen, phosphorus, drought combined (NPD).

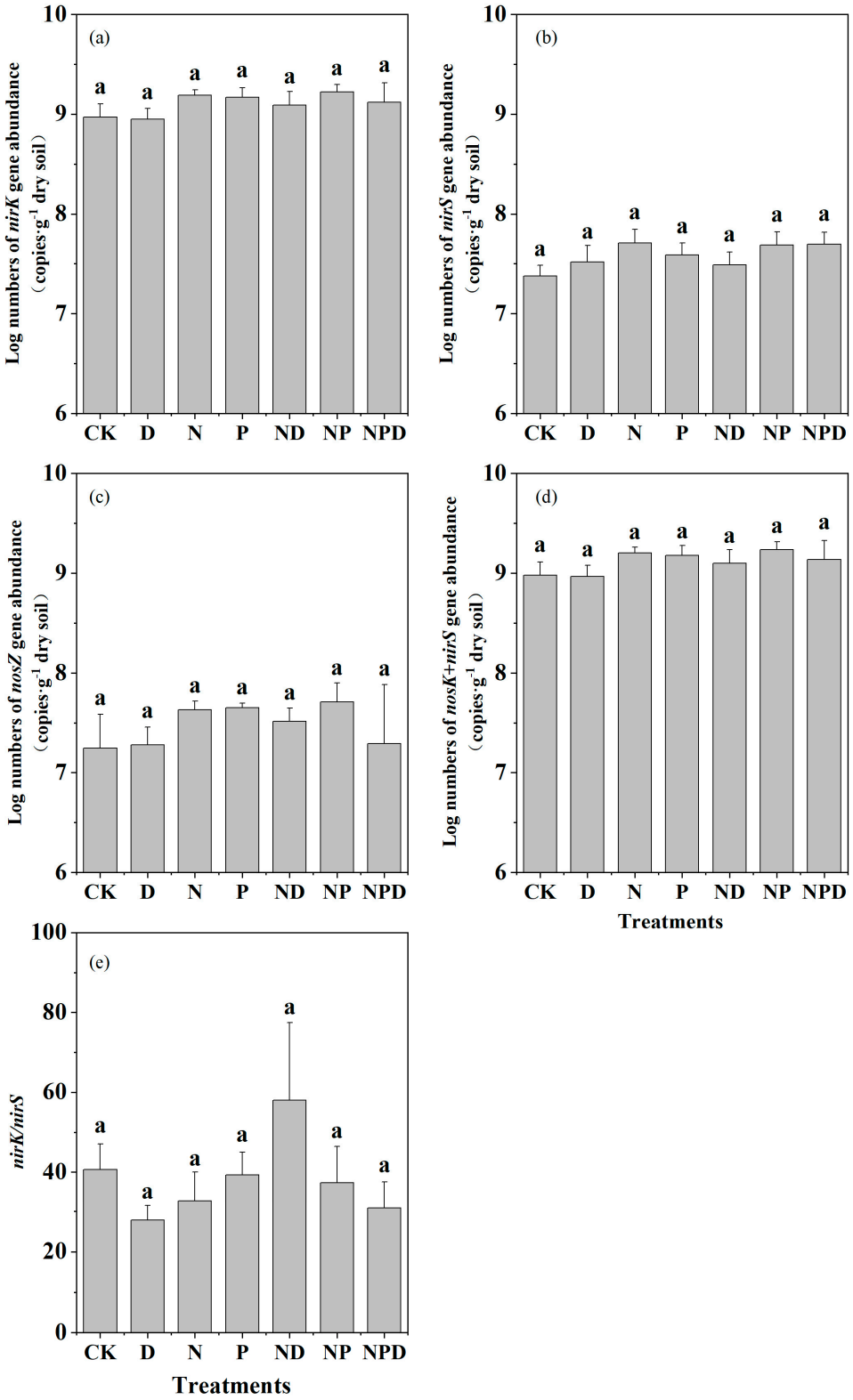

Figure 2. Effects of different treatments on the abundances of the (**a**) *nirK* gene; (**b**) *nirS* gene; (**c**) *nosZ* gene; (**d**) *nirK* gene + *nirS* gene; and (**e**) *nirK*/*nirS*. Note: Data are presented as the mean ± SE (n = 4). Different letters above boxplots indicate significant differences ($p < 0.05$) between treatments. Treatments: control (CK), drought (D), nitrogen addition (N), phosphorus addition (P), nitrogen and drought combined (ND), nitrogen and phosphorus combined (NP), and nitrogen, phosphorus, drought combined (NPD).

Table 1. Results of three-way ANOVA of gene abundance.

Treatment	AOA *amoA*	AOB *amoA*	AOA *amoA* +AOB *amoA*	AOA *amoA* /AOB *amoA*	*nirK*	*nirS*	*nirK+nirS*	*nirK/nirS*	*nosZ*
N	n.s.	0.031	0.013	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
D	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N*P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N*D	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N*P*D	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Note: n.s. = not significant, $p > 0.05$. * The interactive effect of treatments. Treatments: nitrogen addition (N), phosphorus addition (P), drought (D).

3.2. Relationships between the Soil Physico-Chemical Properties and the Abundance of Functional Genes

Pearson correlation analysis demonstrated that the abundance of AOA *amoA* was positively related to TON and NO_3^--N but negatively related to soil temperature (ST) ($p < 0.05$). AOB *amoA* abundance had a significantly positive correlation with NO_3^--N ($p < 0.01$) and NH_4^+-N ($p < 0.05$). The abundance of nitrification genes (AOA *amoA* + AOB *amoA*) was significantly positively correlated with NH_4^+-N ($p < 0.05$) and NO_3^--N ($p < 0.01$). Among the denitrification functional genes, *nirS* gene abundance and NO_3^--N showed a significant positive correlation ($p < 0.05$). The ratio of ammonia-oxidizing archaea to bacteria, the numbers of *nosZ*, *nirK*, and *nirS* + *nirK*, and the ratio of *nirK* to *nirS* abundance had no significant correlation with these environmental factors (Figure 3).

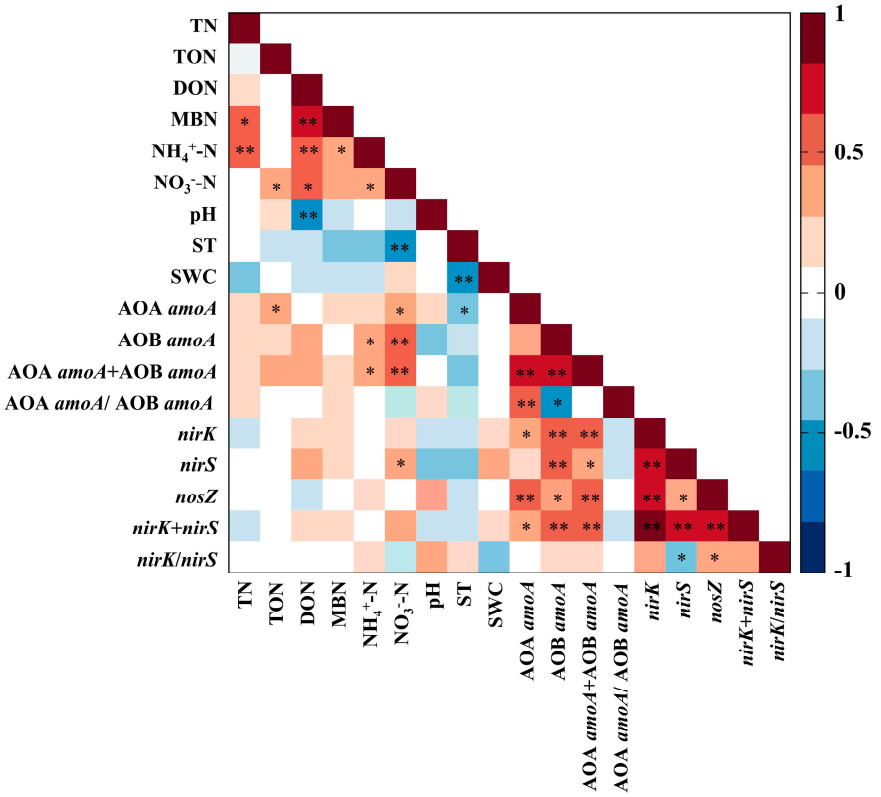

Figure 3. Pearson correlation between gene abundance and soil physical and chemical properties. Note: * Significant at the 0.05 probability level; ** Significant at the 0.01 probability level.

3.3. Main Influencing Factors of Gene Abundance

Mixed linear model analysis results showed that AOA *amoA* abundance was positively affected by pH ($p < 0.1$) and that AOB *amoA* abundance was significantly related to NO_3^--N,

pH, and ST. AOB *amoA* abundance was positively affected by ST ($p < 0.05$) and NO_3^--N ($p < 0.001$), while pH was negatively affected ($p < 0.01$). The nitrification gene abundance (AOA *amoA* + AOB *amoA*) was positively affected by NO_3^--N ($p < 0.001$). The ratio of ammonia-oxidizing archaea to bacterial abundance was positively affected by pH ($p < 0.05$) (Figure 4). The abundance of *nirS* was significantly affected by pH and NO_3^--N, negatively affected by pH ($p < 0.05$) and positively affected by NO_3^--N ($p < 0.05$). The ratio of *nirK* to *nirS* abundance was influenced by soil pH, DON, and ST ($p < 0.05$), and all had positive effects. The abundances of the *nosZ* and *nirK* genes were not significantly affected by these factors (Figure 5).

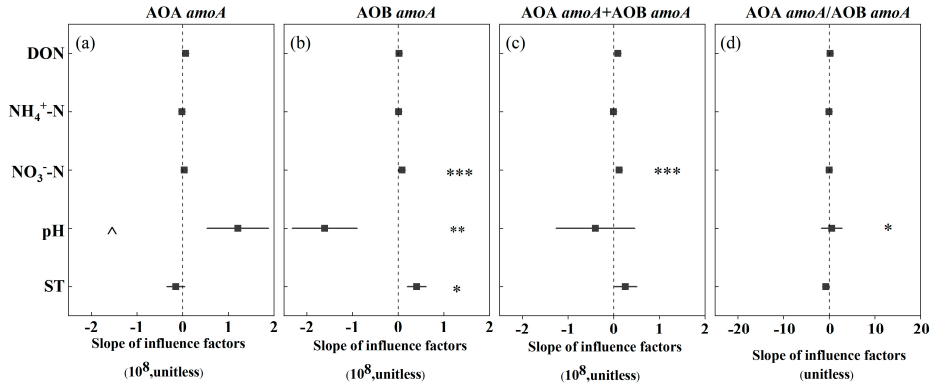

Figure 4. Slope of influencing factors of the (**a**) AOA *amoA* gene; (**b**) AOB *amoA* gene; (**c**) AOA *amoA* gene + AOB *amoA* gene; and (**d**) AOA *amoA*/AOB *amoA*. Note: ^ Significant at the 0.1 probability level; * Significant at the 0.05 probability level; ** Significant at the 0.01 probability level; *** Significant at the 0.001 probability level.

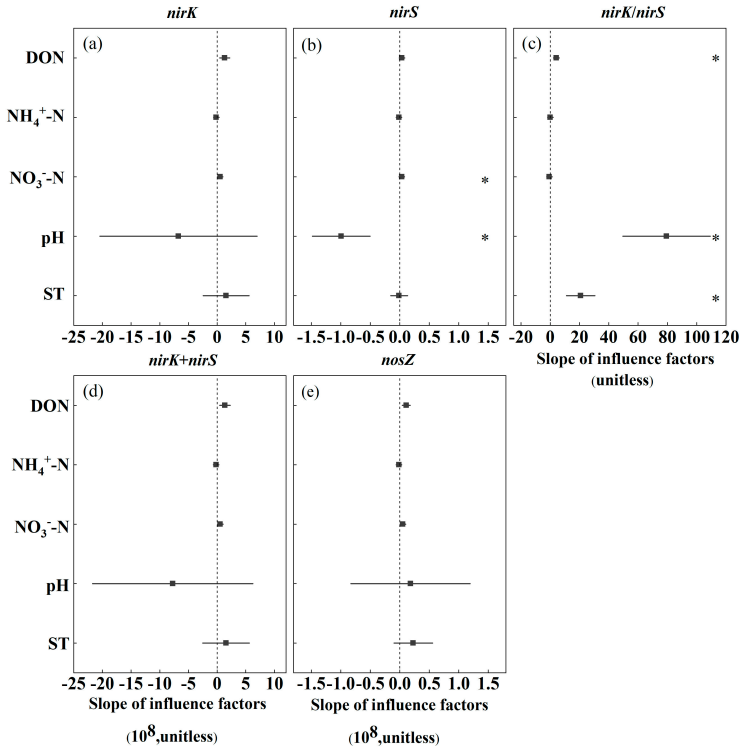

Figure 5. Slope of influencing factors of the (**a**) *nirK* gene; (**b**) *nirS* gene; (**c**) *nirK*/*nirS*; (**d**) *nirK* gene + *nirS* gene; and (**e**) *nosZ* gene. Note: * Significant at the 0.05 probability level.

4. Discussion

4.1. Responses of Nitrification Functional Genes to Changes in Three Resources

N addition induced an increase in the total number of ammonia-oxidizing bacteria and archaea (Figure 1) and significantly affected AOB *amoA* abundance (Table 1) but did not significantly affect AOA *amoA* abundance. This is consistent with the research results showing that AOB *amoA* abundance increases with N application, while the abundance of AOA *amoA* stays relatively stable [14]. This confirms the view that AOB *amoA* is sensitive to high soil nitrogen availability, whereas AOA *amoA* has a weak response to N availability [43,44]. This could be explained because AOA *amoA* is physiologically more adaptable to low concentrations of amino substances [45], whereas AOB *amoA* may be favored by a higher N level [46]. This indicates that the increase in N clearly led to an increase in the population size of the ammonia oxidizers [31] and that AOB *amoA* was more sensitive to N fertilization. The abundance of AOB *amoA* in the NPD-treated soil was significantly more than that in CK soil (Figure 1), while the results of three-way ANOVA showed that N addition, P addition, and drought had no significant interaction effect (Table 1), indicating that N had a more significant promotion effect on AOB *amoA* abundance in the case of P increase and water decrease.

It has been reported that the application of P can accelerate N mineralization and ammonia oxidation [6], alleviate phosphorus limitation and create a more suitable environment for denitrification bacteria [15]. It has also been noted that the addition of P/NP can reduce the abundance of AOA *amoA* in temperate steppes [47] and alpine meadows [48]. In this study, however, the addition of phosphorus alone or in combination did not significantly affect the gene abundance. This may be because P fertilization alone is not sufficient to cause changes in gene abundance, and the effect of P application on the abundance of functional genes is overwhelmed by an increase in N when N and P are added simultaneously [4].

Compared with that of the CK, the nitrification functional gene abundances in the D-treated soil decreased, but it was not significant. This may be attributed to the fact that the wetter the ecosystem is, the weaker the impact of drought [18], and the study area is an alpine meadow in the Zoige Wetland, so it is less affected by drought. In addition, N addition may exacerbate the effects of drought by improving plant productivity [20], which was confirmed by the fact that the AOA *amoA* abundance and AOA + AOB abundance in D-treated soil were significantly less than those in N-treated soil. We observed that although the difference was not significant compared with the control, D-treated and its combination treatments reduced AOA/AOB to less than 1, indicating that drought altered the relative importance of AOA *amoA* and AOB *amoA* and made AOB *amoA* play a more critical role in the ammoxidation process. The variation in gene abundance was explained by substrate concentration, including NO_3^--N and NH_4^+-N, and environmental factors, including pH, ST, and DON (Figure 6). N and its combination treatment acidified the soil and increased the nitrification substrate NH_4^+-N (Table S1), and AOB *amoA* abundance was negatively correlated with pH, so it increased significantly. Different treatments reduced ST and pH overall (Table S1), and AOA *amoA* abundance did not change significantly due to the positive and negative effects of pH and ST offset (Figure 6) or because it was insensitive to environmental changes. The significantly positive relationship between the nitrogen content (NH_4^+-N and NO_3^--N) and the abundance of nitrifying genes (AOA *amoA* + AOB *amoA*) suggested that nitrogen content was an important reason for the increase in nitrification genes [16]. It has been reported that soil pH may determine the relative importance of ammonia-oxidizing archaea and bacteria [15] and that N fertilization can affect the abundance of ammonia-oxidizing bacteria and archaea by changing the soil pH by acidifying the soil [45]. In a nitrogen addition study of alpine meadows, AOB *amoA* was negatively correlated with pH [14], which was in agreement with the research results. In the research results, AOA *amoA* abundance was positively correlated with pH, but the gene abundance did not change significantly, which may indicate that AOB *amoA* was more active and sensitive than AOA *amoA* in alpine meadows.

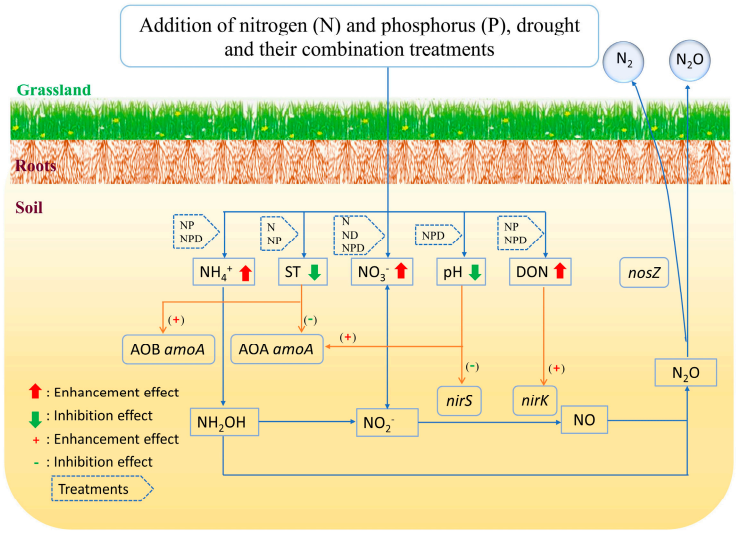

Figure 6. Conceptual diagram of gene abundance response to N addition, P addition, drought, and their combination. Note: NH_4^+(mg/kg): NH_4^+-N content; ST (°C): soil temperature; NH_3^-(mg/kg): NO_3^--N content; DON (mg/kg): dissolved organic nitrogen.

4.2. Responses of Denitrification Functional Genes to Changes in Three Resources

The results showed that the different treatments did not significantly affect *nirK*, *nirS*, and *nosZ* gene abundances. Similar results were reported in another study of N application in an alpine grassland on the Tibetan Plateau [11]. It was also confirmed that the abundance of *nirK* and *nirS* did not change with N and P addition [14]. Notably, we found that the ratio of *nirK* to *nirS* was greater than 20 in all the treated samples, and there were no distinct results among the different treatments, indicating that *nirK* was dominant in alpine grassland and was not affected by environmental changes. These results indicated that denitrification genes could better adapt to future alpine meadow resource changes under N and P subsidence and drought.

The negative effect of pH on *nirS* abundance, the positive effect of NO_3^--N on *nirS* abundance, and the increase in the denitrification substrate NO_3^--N could theoretically increase the abundance of denitrification genes. However, the analysis results suggested that the abundance of denitrification functional genes did not change significantly, possibly because the substrate concentration reduction and gas loss during denitrification offset the above possible increase or because special anaerobic soil conditions are key factors adjusting the copy numbers of the *nirS*, *nirK*, and *nosZ* genes [11]. This could also be due to the short duration of the experiment. A meta-analysis has shown that the susceptibility of functional genes to N and P fertilization and precipitation change is positively correlated with experimental time [33], and some reports have clearly indicated that it takes at least eight [49] or even ten years [50] for microorganisms to produce a significant response to fertilization. Denitrification bacteria are positively influenced by DON, which may be attributed to the fact that most denitrification bacteria are heterotrophic and dependent on organic matter.

4.3. Differences in the Response of Functional Genes to Environmental Changes

The sensitivity of ammonia-oxidizing archaea and bacteria to N addition was different. The results showed that AOB *amoA* was more responsive to N substrate availability than AOA *amoA*, which was consistent with the results of some soil samples from grassland, farmland, and forest ecosystems [16,32]. A meta-analysis showed that AOB *amoA* was more responsive to N increase than AOA *amoA* [31], and another study on the alpine grassland of the Tibetan Plateau also showed that AOB *amoA* plays a leading role in the ammonia oxidation process [11]. This could be due to the viewpoint that AOB *amoA* and

AOA *amoA* occupy different ecological niches [46]. On the other hand, ammonia-oxidizing archaea have a high affinity for ammonia and can tolerate environments with high or low N contents [31]. AOB *amoA* has a higher ribosome content [51], so it may be more suitable for a nutrient-rich environment, and an appropriate amount of N addition can make it a more active and competitive [16].

The nitrifier and denitrifier abundances responded differently to environmental changes caused by different treatments. The results suggested that the reaction of nitrifying functional genes was more significant than that of denitrification functional genes, which might be related to the aerobic or anaerobic nature of the functional genes and the steps they participated in [16,52]. In most cases, ammoxidation is an aerobic process [53], and denitrification bacteria are anaerobic [54], so denitrification bacteria are more adaptable to the anaerobic environment of alpine meadows. AOA and AOB participate in the first step of the nitrification process and are directly affected by the increase in substrate concentration. Nitrate is the first substrate of denitrification, while *nirK* and *nirS* participate in the second step of denitrification. Due to the processes of plant absorption and leaching, the substrate concentration decreases [55], which may buffer the impact of NO_3^--N on *nirK* and *nirS* abundances [56]. Moreover, *nosZ* participates in the last step of denitrification and can adapt to various soil environments better than other genes [57], and because of the emission of NO and N_2O, *nosZ* is less affected by environmental factors.

5. Conclusions

Nitrogen addition, phosphorus addition, drought, and their combined treatments affected gene abundance by changing the substrate concentration (NO_3^--N, NH_4^+-N) and environmental factors (pH, ST, DON) (Figure 6). N addition significantly affected AOB *amoA* abundance and multiplied the total abundance of AOA + AOB. NPD significantly increased AOB *amoA* abundance, but all treatments showed no significant effect on denitrification functional gene abundances. N and P fertilization and drought had no interactive effect on gene abundance. Nitrifying genes were more responsive to environmental changes than denitrification genes, and AOB *amoA* was more responsive to environmental changes than AOA *amoA*. The differences in AOA *amoA* and AOB *amoA* are attributed to their different ecological niches and affinities for ammonia substrates. The differences in nitrifying genes and denitrification genes are mainly related to their aerobic or anaerobic nature and the steps involved in the nitrification and denitrification processes. The results indicated that ammonia-oxidizing bacteria might play a more critical role in soil microbial nitrogen cycling in alpine meadows, and denitrification functional genes can better adapt to environmental changes caused by intensified N and P deposition and reduced rainfall in alpine meadows in the future. The interactive effects of various resource changes on the abundances of N-cycling functional genes and the responses of functional gene communities and structures under future global environmental changes need to be further studied.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13041041/s1, Table S1: Effects of different treatments on soil physical and chemical properties.

Author Contributions: Conceptualization: Y.L.; Methodology: D.T.; Investigation: H.L., J.Z. and J.D.; Data curation: H.L., J.Z. and J.D.; Writing—Original Draft: H.L.; Writing—Review & Editing: H.L., J.Z., D.T. and Y.L.; Visualization: H.L.; Supervision: Y.L.; Funding acquisition: Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Second Tibetan Plateau Scientific Expedition and Research Program (STEP), grant number 2019QZKK0608; National Natural Science Foundation of China, grant number 31770519.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

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