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Grazing Intensity Has More Effect on the Potential Nitrification Activity Than the Potential Denitrification Activity in An Alpine Meadow

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Abstract: On the Qinghai–Tibet Plateau, nitrogen (N) cycling, such as nitrification and denitrification, in the alpine meadow soils have been considerably affected by grazing, with possible consequences for nitrous oxide (N_2O) emissions. However, there is a lack of understanding about how the potential nitrification activity (PNA) and the potential denitrification activity (PDA) might be affected by the grazing intensity. We collected the soil samples in alpine meadow in the east of the Qinghai–Tibet Plateau that was grazed at different intensities from 2015 in peak growing season 2021. We determined the soil physical and chemical properties, the functional gene abundances of nitrifiers and denitrifiers, and the soil PNA and PDA to explore the relationships between a range of abiotic and biotic factors and the PNA and PDA. We found that the PNA and the nitrifiers were significantly affected by the grazing intensity but that the PDA and the denitrifiers were not. The ammonia-oxidizing archaea (AOA) abundance was highest but the ammonia-oxidizing bacteria (AOB)abundance was lower than the control significantly at the highest grazing intensity. The AOA abundance and the soil NH_4^+-N explained most of the variation in the PNA. The pH was the main predictor of the PDA and controlled the *nirS* abundance but not the *nirK* and *nosZ* abundances. Overall, the PNA was more responsive to the grazing intensity than the PDA. These findings can improve estimations of the nitrification and denitrification process and N_2O emissions in alpine meadow.

Keywords: nitrification and denitrification; N-cycling; functional gene; grazing; Tibetan alpine grassland

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1. Introduction

Grazing, a global land management practice, has widespread social and environmental impacts [1]. The grassland area of the Qinghai–Tibet Plateau is the largest in Eurasia [2], and alpine meadow accounts for a large part (about 85%) of this pasture [3]. The integrity of alpine meadow is threatened by overgrazing [4], and reports have proved that more than half of the alpine meadow is degraded [5,6], which has consequences for nitrogen (N) cycling rates in the soil and nitrous oxide (N_2O) emissions [7]. Of the N-cycling processes, both nitrification and denitrification generate N_2O [8–11]. In the soil, the potential nitrification activity (PNA) and potential denitrification activity (PDA) are frequently described by the functional genes of nitrification (ammonia-oxidizing archaea (AOA) and bacteria (AOB)), and denitrification (*nirS*, *nirK*, and *nosZ*) [12–17]. We could increase our understanding of how the soil PNA and PDA might respond to grazing in these alpine meadows by using an N-cycling model that incorporates these functional genes.

The PNA and PDA in the soil are carried out by nitrifiers and denitrifiers, respectively, and are controlled directly or indirectly to some extent by the soil abiotic factors. To date,

there is a lack of agreement about what controls the PNA and PDA in soil. For example, studies have shown that, in the grazed soil, the soil properties, such as the soil moisture, pH, and inorganic N efficiency, are the dominant predictors for the PNA and PDA [18–20]. These abiotic factors in the soil may change with the grazing intensity, and may influence the PNA and PDA by controlling the microbial activity and the reaction process in the soil [18,21,22]. Other reports have shown that the PNA and PDA are mainly determined by the abundances of the microbial communities [23]. For example, the nitrification rate was more strongly (positively) correlated with the nitrified enzyme activity and the AOB species abundance than with the AOA at a high grazing intensity [7,24]. From research about the denitrification process, Cuhel, et al. [25] thought that the PDA showed a positive correlation with the *nirS* abundance. In contrast, Petersen et al. [26] examined soils from five different ecosystems and showed that the PDA was directly affected by the *nosZ* abundance and indirectly affected by the *nirK* and *nirS* abundances. Zhong, et al. [27] found that, of a range of nitrifying and denitrifying functional genes measured in soil from an intensely grazed pasture, the *nirK* abundances were most closely linked with the potential for N_2O emissions. The results from these studies show that microorganisms with the same function could affect the PNA and PDA in different ways.

The responses of soil PNA and PDA to grazing intensity were also inconsistent across studies. When livestock excrement is introduced to grazed land, the pH, nutrient turnover rate between plants and soil [28], and the substrate concentration for microbes increase [29]. The enzyme activity and the abundance of functional genes are then stimulated [14], giving rise to increases in the nitrification and denitrification rates in the soil [24,30]. These increases have been reported to become more pronounced as the grazing intensity increases [24,28,31]. However, some research has proved that changes in the PNA and PDA were either minimal or negative under intense grazing [18,21]. Shi, et al. [32] suggested that the trampling of livestock might inhibit the diffusion of oxygen through the soil during nitrification, which would not be conducive to the activity of nitrifiers. Xu, et al. [21] reported that the denitrification rate in soil from grazed pasture was affected when the soil available inorganic N decreased. Zhong, et al. [33] found that, since grazing did not cause variations in the availabilities of C and N in the soil significantly, moderate grazing did not induce a significant response in the soil PNA.

In recent studies about the grassland ecosystems of the Qinghai–Tibet Plateau, most research has documented how grazing has affected the vegetation and soil properties [34–37]. However, there is little information about how the functional microbial abundances and the PNA and PDA in soil respond to grazing at different intensities in alpine meadows. To address this gap in knowledge, we set up an experimental area in an alpine meadow in the eastern part of the plateau. We determined the responses of soil physical and chemical properties to the grazing intensity at first. Then, we evaluated the abundances of nitrifiers and denitrifiers by quantitative PCR (qPCR) and explored the driving factors for the variation. In the end, combined with the soil abiotic and biotic factors, we examined how the soil PNA and PDA changed under four different grazing treatments and discussed the mechanism of the change in PNA and PDA. The objectives of our study were (1) to determine the responses of the soil PNA and PDA to the increased grazing intensity and (2) to evaluate the extent to which the soil abiotic and biotic factors explained the variations in the soil PNA and PDA on the Qinghai–Tibet Plateau. We proposed the following hypotheses: (1) the microbial abundances and the potential activities of the nitrification and denitrification process in the alpine meadow would stimulate under the increased grazing intensity, and (2) the soil moisture and N and C availabilities would be the main controls on the potential activities.

2. Materials and Methods

2.1. Site Description and Experimental Design

The study site is in an alpine meadow at the Yak Grazing Intensity Platform of the Sichuan Zoige Alpine Wetland Ecosystem National Observation and Research Station,

in Hongyuan County, Sichuan Province, China, the eastern part of the Qinghai–Tibetan Plateau (102°33′ E, 32°48′ N, 3500 m). The mean annual temperature and the mean annual precipitation is 1.5 °C and 747 mm, respectively, with 80% of the precipitation occurring from May to September. The bulk density and pH of the soil are 0.89 g cm^{-3} and 6.23, respectively [35]. The experimental site was dominated by *Kobresia pygmaea*, *Kobresia humilis*, *Saussurea nigrescens*, *Elymus nutans*, and *Deschampsia cespitosa*.

A 10-hectare pasture was built and the experiment of different grazing intensity began in 2015. The site was divided into 12 plots with fences to facilitate 3 replicates of 4 treatments (Figure S1). The treatments were ungrazed (UG) or control, light grazing (LG, 1 yak ha^{-1}), moderate grazing (MG, 2 yaks ha^{-1}), and heavy grazing (HG, 3 yaks ha^{-1}). Each of the nine grazed plots covered 1 ha, and the other three ungrazed plots together occupied a total of 1 ha. The yaks chosen for the experiment each weighed around 200 kg, to ensure that each yak would graze uniformly, and were kept in the grazing plot throughout the growing season. The experimental site has been described by Mipam, et al. [35] and Mipam, et al. [38] in more detail.

2.2. Soil Sampling and Analyses

On a sunny day in early August 2021, we collected 63 soil samples in the study site by using a corer with 3 cm diameter. To a depth of 0–10 cm, the samples were collected from six randomly selected subplots. Five samples were taken from these randomly selected spots in each grazing plot, and then the five samples were thoroughly mixed to produce a mixed sample. Once collected and mixed, the samples were placed in a chilled incubator for transportation. Before analysis, the soil was sieved through a mesh (2-mm) and separated into 2 sections. One of them was for analyzing the soil abiotic factors and the PNA and PDA, which was kept at −20 °C, and the other part was kept at −80 °C for quantitative PCR analysis.

The soil moisture (SM) was determined with a portable soil moisture meter (SM150 Kit, Delta-T, Britan) in the field during sampling. The soil pH was measured with a pH analyzer (S210-K, Mettler Toledo, Greifensee, Switzerland) by creating a suspension of 10 g soil and 40 mL distilled water. The total nitrogen (TN), organic nitrogen (TON), total carbon (TC), and organic carbon (SOC) concentrations were determined with a CN analyzer (CN802, VELP, Milan, Italy). The NH_4^+-N and NO_3^--N contents of the soil were determined using a flow injection analyzer (Auto Analyzer 3, Bran + Luebbe, Hamburg, Germany) after extraction (10 g of each soil sample) with 0.5 M K_2SO_4 (40 mL). The concentrations of dissolved organic carbon (DOC) were determined using a TOC analyzer (TOC-LCPN, Shimadzu, Kyoto, Japan).

2.3. Soil DNA Extraction and Quantification of Nitrifiers and Denitrifiers

The total DNA was extracted from 0.25 g soil, following the manufacturer's instructions supported by Power Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). A spectrophotometer (Nanodrop Technologies, USA) was used for the checking of the quantity of the DNA, and it was defined as the microbial biomass (MB). By using real-time PCR detection system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA), the AOA, AOB, *nirK*, *nirS*, and *nosZ* abundances were quantified in triplicate in 96-well plates. To create a standard curve, the real-time PCR tests of plasmids containing each target gene were conducted in triplicate at 10-fold serial dilutions. Table S1 describes the primer pairs of each gene and their amplification conditions. Each qPCR reaction solution was 20 µL and contained 10 µL TB Green Premix Ex Taq (TaKaRa, Tokyo, Japan), 1 µL DNA template from soil, 0.2 µL primers, 0.08 µL ROX, and 8.52 µL ddH_2O. The amplification efficiency and R^2 values ranged from 85% to 110%, and 0.995 to 0.999 for all runs, respectively. Tang, et al. [39] showed more details of the qPCR program.

2.4. Potential Nitrification and Denitrification Activities in the Soil

Following the procedure described by Wang, et al. [40], the PNA in the soil was determined. A portion of each sample (10 g) was added to a 250-mL serum bottle, and then incubated at 25 °C for 14 days. The NO_3^--N contents were determined at the start of the incubation and again after 14 days, after extraction with 0.5 M K_2SO_4 (40 mL). The PNA was calculated and expressed as NO_3^--N·d^{-1}·g^{-1} soil.

The PDA of the soil samples was determined following Smith and Tiedje [41]. In a 100-mL anaerobic bottle, the soil samples that were comparable to 4 g dry soil weight were incubated into 20 mL culture medium. This culture solution was a mixture of 288 mg glucose-C, 2 mg chloramphenicol, and 56 mg KNO_3 dissolved in 1 L sterile water. The mixture was homogenized for 20 min to clear the residual air from the soil pores. Then, to create anaerobic conditions and suppress the activity of N_2O-reducatase, the air was evacuated from each anaerobic bottle and replaced with a combination of N_2-C_2H_2 (90:10 *v*/*v*). The gas samples (20 mL) were obtained at the beginning and the end of the 2-h incubation by syringe, and a gas chromatograph (7890A, Agilent, Santa Clara, CA, USA) was used for the determination of N_2O concentrations. The PDA was taken as the difference between the N_2O concentrations before and after incubation, and was calculated as ng N-N_2O h^{-1}·g^{-1} dry soil [42].

2.5. Statistical Analyses

The microbial N turnover potential can be determined from the sum or ratio of the different gene abundances. The (AOA+AOB) represented the NH_4-N oxidation, (AOA+AOB)/(*nirK*+*nirS*) ratio represented the NO_3^--N leaching, (*nirK*+*nirS*) gene abundance indicated gaseous N loss, and the consumption of N_2O was indicated by the (*nirK*+*nirS*)/*nosZ* ratio. Under different grazing intensities, we analyzed the responses of these microbial N turnover potentials from the sums or ratios of the different gene abundances.

Before this analysis, the data distribution was evaluated for normality and homogeneity, and the gene copy counts were log-transformed. One-way ANOVA was used to evaluate the normally distributed data, followed by multiple comparisons using the Duncan's test for homogeneous variance and the Dunnett-t3 test for uneven variance. The Kruskal–Wallis nonparametric test was used to examine the non-normally distributed data that could not be transformed to normality. To study the relationships between the microbial gene abundances and soil properties, we conducted a redundancy analysis (RDA) in Canoco 5. The correlations between the functional gene abundances, soil abiotic variables, and the PNA and PDA were evaluated through the Pearson correlation test. To estimate the dominant factors controlling the PNA and PDA, we performed multiple regression analysis. We used SPSS and set a significance threshold of $p < 0.05$ for these analyses, and GraphPad Prism 8 was used to generate the figures.

The direct and indirect impacts of the soil properties and functional gene abundances on the PDA and the PNA were determined by structural equation modeling (SEM) using AMOS 23.0 (see the SEM in Figure S2). Then, we used the maximum likelihood estimation method to fit the data to the models. The degrees of freedom (*df*), chi-square (χ^2) index, root mean square error of approximation (RMSEA), and its associated *p* value were shown to determine the adequacy of the models. The model fit could be considered good when the *p* value was greater than 0.05, the RMSEA was smaller than 0.05, and the χ^2/df was between 0 and 2 [43].

3. Results

3.1. Effects of the Grazing with Increased Intensity on Soil Abiotic Factors

Differences between the properties of soil from the four grazing treatments were significant (Table 1). The SM varied significantly under the different grazing intensities ($p < 0.05$). It was 19.98% for the control but reached a maximum of 33.44% for the HG treatment. The NH_4^+-N also changed noticeably ($p < 0.05$) and decreased as the grazing intensity increased. The concentrations of NH_4^+-N were 63% less for the HG treatment

than for the UG. However, there were no significant changes in the other soil properties ($p > 0.05$).

Table 1. Main characteristics of the alpine meadow soil under different grazing intensities (mean ± standard errors, $n = 3$).

	UG	LG	MG	HG
SM (%)	19.98 ± 2.8c	26.93 ± 3.44b	31.29 ± 2.17ab	33.44 ± 2.91a
pH	5.89 ± 0.1a	5.85 ± 0.07a	5.89 ± 0.09a	5.84 ± 0.06a
TN (g/kg)	3.72 ± 0.39a	4.1 ± 0.13a	4.14 ± 0.02a	4.15 ± 0.35a
TC (g/kg)	37.64 ± 5.44a	42.62 ± 0.72a	42.36 ± 0.83a	42.35 ± 3.24a
TON (g/kg)	3.56 ± 0.44a	3.75 ± 0.07a	3.78 ± 0.17a	4.04 ± 0.25a
SOC (g/kg)	35.34 ± 5.7a	44.03 ± 7.46a	39.37 ± 3.42a	40.38 ± 3.43a
TC/TN	10.09 ± 0.39a	10.58 ± 0.21a	10.4 ± 0.38a	10.37 ± 0.32a
DOC (mg/kg)	51.32 ± 5.28a	44.36 ± 5.04a	42.43 ± 6.52a	47.41 ± 2.59a
NH_4^+-N (mg/kg)	14.24 ± 3.58a	11.21 ± 0.84a	8.9 ± 2.95ab	5.3 ± 3.04b
NO_3^--N (mg/kg)	6.12 ± 1.92a	5.63 ± 3.31a	8.21 ± 3.76a	10.36 ± 3.01a

UG is ungrazed treatment, LG is light grazing treatment, MG is moderate grazing treatment, and HG is heavy grazing treatment. Different letters indicate significant differences at the 0.05 level between four grazing intensities.

3.2. Effects of the Grazing with Increased Intensity on Biotic Factors

The soil MB varied, but not significantly, among the different treatments, and the MB was higher in the LG treatment and lower in the MG and HG treatments than in the UG. The changes in the functional gene abundances followed different patterns for nitrification and denitrification (Figure 1). The abundances of the microbial genes involved in nitrification varied significantly among the four grazing intensities ($p < 0.05$). Compared to the control group, the AOA abundance was lower in the LG but was significantly higher in the MG and HG. The AOB abundance was significantly lower under the three grazing treatments than in the UG treatment. The abundances of the denitrification genes were not significantly affected by the grazing intensity ($p > 0.05$).

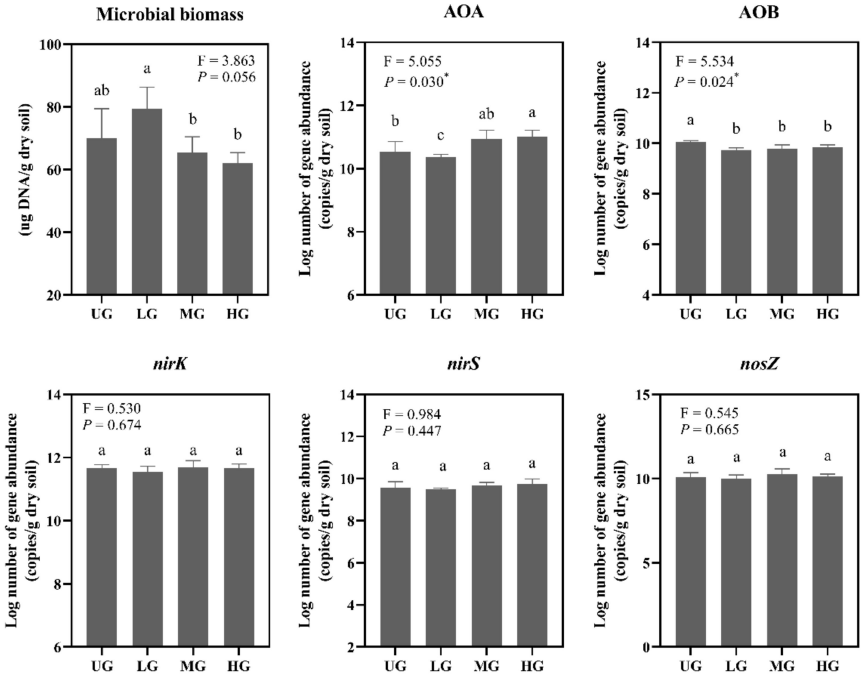

Figure 1. Microbial biomass (MB) and the abundances of the ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), *nirK*, *nirS*, and *nosZ* functional genes for the control and the three different grazing intensities. The bars represent the standard error ($n = 3$). Different letters indicate significant differences at the 0.05 level. UG is ungrazed, LG is light grazing, MG is moderate grazing, and HG is heavy grazing. * indicates significance at the 0.05 probability level.

Relative to the control, the (AOA+AOB) was lower for the LG but was significantly higher for the MG and HG ($p < 0.05$). The AOA abundances were significantly more abundant than the AOB in the MG and HG treatments ($p < 0.01$). The ratio of (AOA+AOB)/(*nirK*+*nirS*) was significantly higher for the MG and HG than for the UG and LG. The (*nirK*+*nirS*), *nirK*/*nirS*, and (*nirK*+*nirS*)/*nosZ* were similar for the different treatments ($p > 0.05$) (Figure 2).

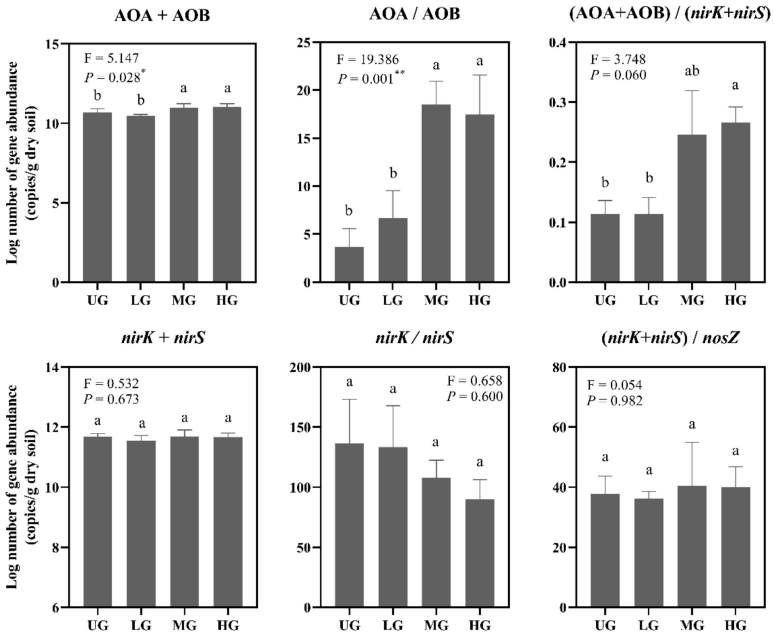

Figure 2. Indexes of the microbial N turnover potential at different grazing intensities. The bars represent the standard error ($n = 3$). Different letters indicate significant differences at the 0.05 level. UG is ungrazed, LG is light grazing, MG is moderate grazing, and HG is heavy grazing. * indicates significance at the 0.05 probability level. ** indicates significance at the 0.01 probability level.

3.3. Soil PNA and PDA at the Different Grazing Intensities

The PNA and PDA in the soil responded differently to the four grazing intensities (Figure 3). The soil PNA increased as the grazing intensity increased ($p < 0.05$). The soil PNA was about 83%, 113%, and 411% higher in the LG, MG, and HG treatments, respectively, than in the control. In contrast, the soil PDA ranged from 131.42 to 175.13 ng $N\text{-}N_2O$ $h^{-1}{\cdot}g^{-1}$ dry soil, and did not vary among the different grazing intensities significantly ($p > 0.05$).

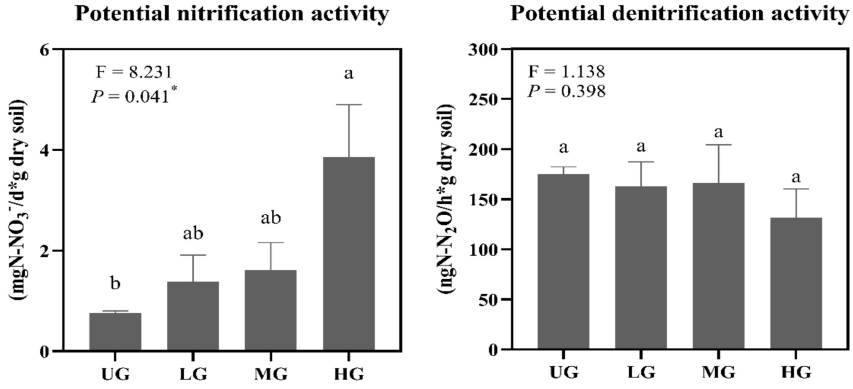

Figure 3. The potential nitrification (PNA) and denitrification activities (PDA) at different grazing intensities. The bars represent the standard error ($n = 3$). Different letters indicate significant differences at the 0.05 level. UG is ungrazed, LG is light grazing, MG is moderate grazing, and HG is heavy grazing. * indicates significance at the 0.05 probability level.

3.4. Contribution of the Abiotic and Biotic Factors to the Changes in PNA and PDA

A total of 88.84% of the variations in the functional gene abundances were explained by the two RDA axes in the alpine meadow (Figure 4). Of the different variables, the NO_3^--N and SM explained most of the variations in the nitrifiers and denitrifiers in the four grazing treatments, and explained 53.4% ($p < 0.01$) and 17.0% ($p = 0.03$) of the variations, respectively.

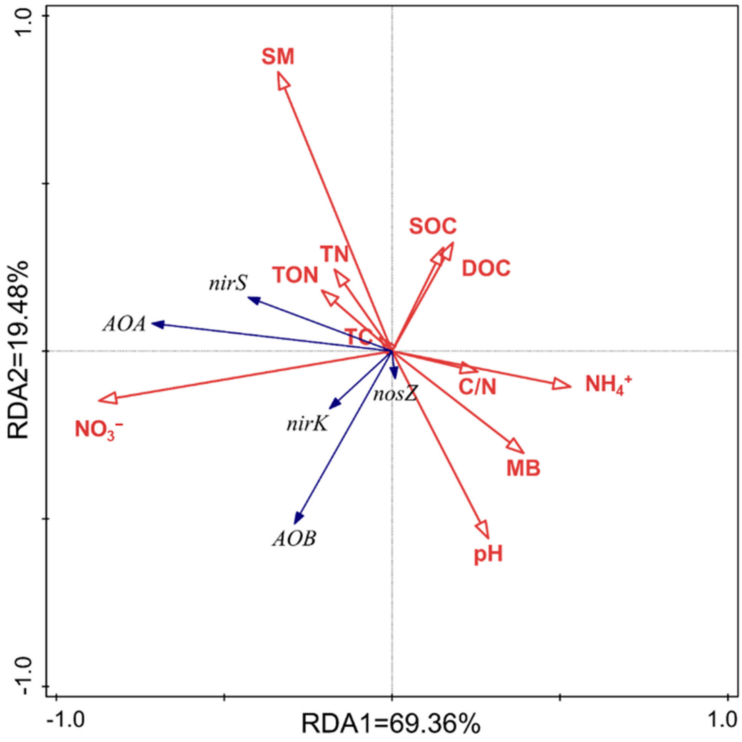

Figure 4. Redundancy analysis (RDA) of the soil properties and functional gene abundances. The soil properties and functional genes are indicated by red and blue lines, respectively.

The AOA abundance was correlated positively with the SM and NO_3^--N, and was correlated negatively with the NH_4^+-N and MB ($p < 0.05$). The abundance of AOB was correlated with the SM negatively and was correlated with the NO_3^--N positively ($p < 0.05$). The sum of the nitrifier abundances had positive correlations with SM and NO_3^--N but had negative correlations with the pH, NH_4^+-N concentration, and MB ($p < 0.05$).

For the microbial genes involved in denitrification, the abundance of *nirS* showed significant relationships with the SM, pH, and the concentration of NO_3^--N ($p < 0.05$), while the abundances of *nirK* and *nosZ* did not show significant correlation with the soil properties ($p > 0.05$). The ratio of *nirK*/*nirS* correlated with the pH and NH_4^+-N positively but correlated with the SM, NO_3^--N, and DOC negatively ($p < 0.05$). The (AOA+AOB)/(*nirK*+*nirS*) ratio showed positive correlation with the SM, TN, and NO_3^--N, and negative correlation with the C/N ratio, NH_4^+-N, and MB ($p < 0.05$). The PNA was negatively correlated with NH_4^+-N ($p < 0.05$), and the PDA was not significantly correlated with any of the soil properties ($p > 0.05$) (Figure 5).

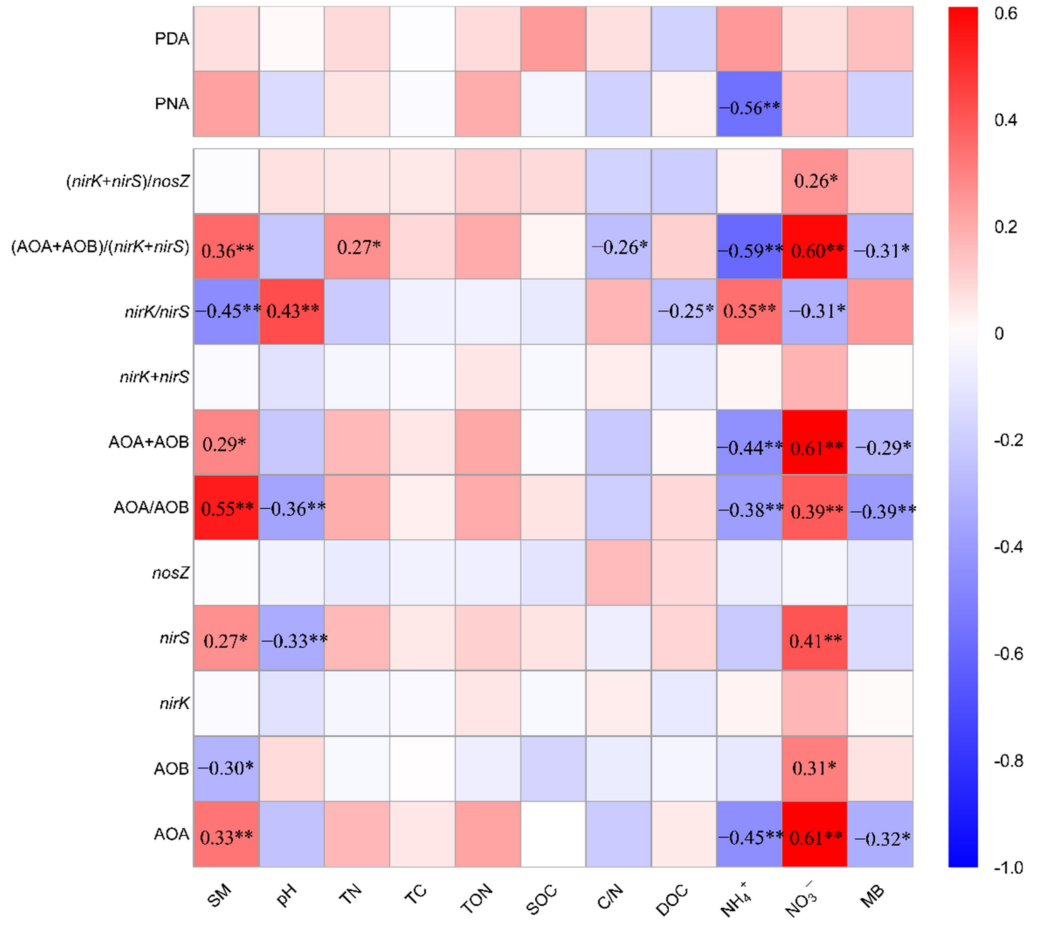

Figure 5. Correlation coefficients between the soil properties, functional gene abundances, PNA, and PDA. * indicates significance at the 0.05 probability level. ** indicates significance at the 0.01 probability level.

The multiple regression analysis indicated that different factors influenced the PNA and PDA (Table 2). The functional gene abundances of AOA and the NH_4^+-N concentration explained 55.0% of the variation in soil PNA. The soil pH explained 94.3% of the variation in the PDA for the four grazing intensities. The standardized Beta values show that, except for NH_4^+-N, there were positive correlations between the explanatory factors and the dependent variables.

Table 2. The relative importance of the biotic and abiotic factors for the PNA and PDA in the soil, determined by multiple regression analysis.

	Factor (n = 63)	Standardized Beta	p	ss%
PNA	AOA	1.22	<0.001	45.4
	NH_4^+-N	−0.63	<0.001	9.6
	Total explained			55.0
PDA	pH	0.97	<0.001	94.3
	Total explained			94.3

ss% is the sum of squares contributed by each parameter.

We used an SEM for a more detailed analysis of the biotic and abiotic influences on the PNA and PDA (Figure 6). The grazing intensity had direct and significant impacts on the SM and NH_4^+-N, and also affected the AOA and AOB abundances. The combination of TON and NO_3^--N caused an increase in the PNA. The PDA was regulated directly by the grazing intensity. While the pH and SM had no direct effect on the PDA, they indirectly affected the PDA through changes in the *nirS* abundances. The PDA was also affected

by *nirK* through changes in the *nirS* and *nosZ* abundances. We found that the PNA and PDA were affected by the grazing intensity indirectly through a combination of abiotic and biotic factors.

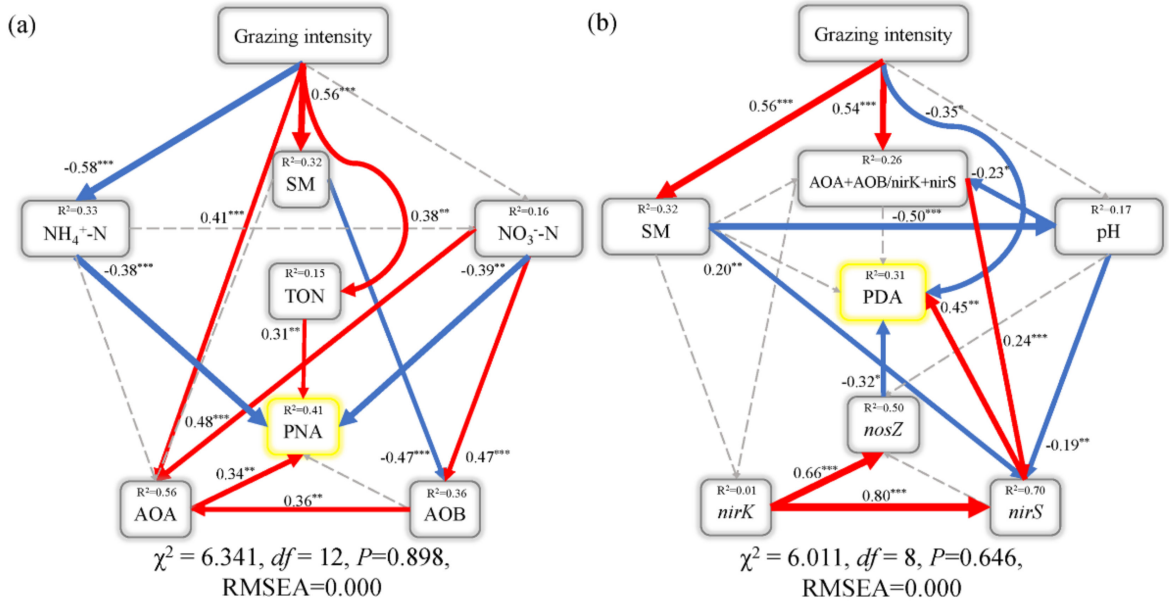

Figure 6. The structural equation model (SEM) of the relationships between the (**a**) PNA, (**b**) PDA, and the potential influences in an alpine meadow. The red, blue, and dashed lines indicate positive, negative, and path coefficients, respectively. The thickness of the lines represents the magnitude of the path coefficient (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The R^2 values indicate the degree of variance explained. Goodness-of-fit statistics are shown underneath the models.

4. Discussion

4.1. How the Grazing Intensity Affected the Abiotic Factors, and the Implications for the PNA and PDA

Studies have shown that grazing led to a reduction in the SM [44,45], and the reduction was quite obvious in arid regions [46]. Alpine meadows are not arid and receive abundant precipitation [36], and the samples were collected during the rainy season. Soil in grazed pasture is frequently compacted; as the grazing intensity increases, the soil becomes increasingly compacted, meaning that less soil water is lost through evapotranspiration [47,48]. Moreover, the yak consumed the vegetation, thereby reducing the plant water requirements and transpiration, which would cause the SM to increase [49–51]. Some studies have shown a decrease in PNA and an increase in PDA along with the SM increase [32,52], which contrasts with our findings. Here, it may not be possible to clearly see the effects of SM on the PNA and PDA, as the effects of SM may be combined with the effects of other factors. We found that the decline in the abundance of AOB and the promotion in the abundance of AOA were related to the SM, and that these changes caused an increase in the soil PNA. At this study site, SM had a positive influence on the PDA in soil under grazing after corresponding changes in the soil pH and *nirS* abundance. These results indicate that the SM might have directly and indirectly influenced the microbial abundances through regulating the soil oxygen concentrations and pH [53], which then influenced the soil PNA and PDA.

The NH_4^+-N concentrations were highest in the control and decreased as the grazing intensity increased, which is consistent with the findings from Enriquez, et al. [54]. This finding suggests that N mineralization, which is closely related to soil microbes [55,56], was suppressed under heavy grazing at this site [57,58]. The MB also decreased under grazing, and this reduction was not conducive to soil organic N mineralization to inorganic N [59,60]. The yak feeding also meant that the litter was depleted, with the result that the

N in the plants did not return to soil to be mineralized [57]. In contrast to our findings, studies elsewhere have shown positive correlations between the PNA and the NH_4^+-N concentrations [39,61]. This could help explain why the NH_4^+-N did not influence the PNA strongly under grazing, and shows that the AOA abundance was more important as a positive coregulator of the PNA. Studies have proved that the increase in the PNA observed in grazed pasture is related to changes in the abundance of the soil nitrifiers in grasslands [23,62].

The pH was the dominant abiotic control on the PDA, and, as also reported by Zhong, et al. [33], the PDA did not show significant variation under grazing relative to the control. There was no obvious change in the pH at the study site [63,64], yet researchers elsewhere reported that the soil pH would change when the grazing intensity increased [65,66]. The different results may reflect the frequent and high rainfall in the alpine meadow that buffers any changes in the pH caused by the grazing [67], which then affects the soil PDA. Moreover, the SEM model showed that the pH could affect the PDA indirectly through the abundance of *nirS*, but the change in *nirS* abundance was not significant. As Cuhel, et al. [25] stated, if the pH changed, the enzyme activities and the abundances of the denitrifiers would only change over the long term; if this occurred, it could then affect the PDA. Moreover, the alpine meadow soil is acidic and the grazing intensity had no effect on the pH of the soil at the study site [64]. Here, the denitrifying microbial functional group and the PDA did not respond significantly to the changes in the external environment.

4.2. How the Grazing Intensity Affected the Biotic Factors, and the Implications for the PNA and PDA

For the nitrifiers, the abundance of AOA was higher than that of AOB significantly, which is consistent with the finding from Egan, et al. [68]. Microorganisms with the same functions responded differently to the SM and NH_4^+-N availability at different grazing intensities. The results suggest that the AOA and AOB microbes filled different niches. Compared to the higher substrate affinity of ammonia monooxygenase encoded by AOA, that of AOB was lower. It means that the microbial activity of AOB will be better suited to the soil environments that have higher pH value and greater ammonium contents [69,70]. In contrast, its counterpart, the ammonia monooxygenase encoded by AOA, could reach saturation at low NH_4^+-N concentrations. Studies have shown that AOA is more dominant than AOB in the nitrification process under low N concentrations in acidic soils [12,15], which agrees with the corresponding increases in the abundance of AOA and PNA under different grazing intensities at the study site. Additionally, the SM increased under the increased grazing intensity, whereas the soil oxygen concentration decreased. AOA require less oxygen to participate in nitrification than AOB, and AOA can survive in an anaerobic environment [71]. These contrasting responses of the AOA and AOB ensured that the nitrification and the PNA continued, and even increased as the grazing intensity promoted.

For the denitrifiers, the *nirK*, *nirS*, and *nosZ* abundances did not change noticeably in response to the grazing intensity. This is consistent with the finding of Yin, et al. [24], who also reported that different grazing intensities had a limited impact on the denitrifiers. In arid regions where ecosystems are sensitive to precipitation [72,73], the functional gene abundances of the denitrifying microorganisms and the PDA were closely associated with the SM [20,61]. Here, the *nirK*, *nirS*, *nosZ*, and the PDA in this alpine meadow soil were not significantly correlated with the increases in the SM as the grazing intensity increased. The anaerobic environment in the alpine meadow soil, caused by the high precipitation, is already suitable for denitrifiers and the denitrification process [20]. In addition, the denitrification functional community and its activities are sensitive to the N and C availabilities in the soil [74,75]. The SOC and TN can promote the growth and physiological activities of heterotrophic denitrifying bacteria, leading to a higher denitrification rate [76]. As substrate, the NO_3^--N is also crucial for the denitrifiers and the soil denitrification rate [75,77,78]. The SEM showed that *nirK* affected the soil PDA through the changes in *nirS* and *nosZ*. Nevertheless, these soil abiotic factors were only slightly

affected by the grazing intensity, but the changes in the environment were not sufficient to noticeably affect the abundances of the denitrifying genes and the PDA [26,33].

4.3. The Grazing Intensity Had More Effect on the Variables That Influenced the PNA Than on Those That Influenced the PDA

The PNA was stimulated by the grazing intensity, and the findings from the correlation analysis, multiple regression analysis, and the SEM model suggested that the abundance of AOA and, in turn, the PNA would be controlled by the SM and the NH_4^+-N availability. Nitrification is an aerobic process with NH_4^+-N as the substrate [10]. Studies have reported that the PNA would increase with increases in NH_4^+-N [39]. The soil permeability decreased and the diffusion of oxygen was inhibited because of the trampling by livestock, which, in turn, caused a decline in the nitrification activity in the grazed plots [18,32]. Researchers have also suggested that the nitrification process is mainly determined by the AOB [24,79]. However, in this alpine meadow, even if the soil NH_4^+-N and the oxygen concentrations declined as the SM increased, the PNA responded positively to the grazing intensity. These results indicate that the nitrifier abundances, especially AOA, were a better predictor of the PNA than the abiotic factors in the alpine meadow pasture. Some studies also reported that, in acidic soils, the PNA was mainly determined by the AOA [80,81]. Further, an incubation experiment conducted by Kraft, et al. [82] found that AOA could produce oxygen for nitrification in oxygen-depleted conditions. This means that AOA may be abundant in an environment where the oxygen level is very low or undetectable [83,84].

The increase in the AOA+AOB abundance and the minimal changes in the three denitrifier abundances caused the (AOA+AOB)/(*nirK*+*nirS*) to increase, and the increases were especially pronounced under the MG and HG treatments. While the soil exchangeable NO_3^--N decreased (insignificantly), the NO_3^- leaching potential increased along with the grazing intensity, as indicated by the (AOA+AOB)/(*nirK*+*nirS*), and might reflect a high concentration of N uptake by aboveground plants and inevitable leaching in wet soil during the rainy season [39]. In these soil conditions, the PNA in the soil could increase as the grazing intensity increases.

While there is some debate about whether abiotic factors or biotic factors are more important for the PDA [85–87], most researchers conclude that abiotic factors are more reliable predictors of the PDA than biotic variables [52,88]. *NirK* and *nirS* encode nitrite reductase, and *nosZ* encodes nitrous oxide reductase. By regulating the activities of nitrite reductase and nitrous oxide reductase, the soil pH can directly influence the response of PDA to the increased grazing intensity [61,89,90]. The soil pH can also control the PDA indirectly by influencing the abundances and diversity of denitrifiers [19,91]. Our SEM showed that the increases in the SM suppressed the potential for changes in the soil pH as the grazing intensity increased [67]. The pH mainly affected the PDA through influencing the *nirS* abundance. In this experimental site, the alpine meadow soil was acidic, and the *nirK* abundance was much higher than the *nirS* and *nosZ* abundances. As shown by Bowen, et al. [92] and Herold, et al. [93], the number of *nirK* communities increased as the soil pH decreased, and the *nirS* and *nosZ* communities showed positive correlation with the pH. These findings indicate that the soil environment in this study was already suitable for the physiological and metabolic activities of the *nirK*-denitrifiers, and that *nirS* was suppressed by the soil acidity [90]. However, the *nirS*-denitrifiers could make an important contribution to the denitrification process if the pH was near-neutral [94]. The PDA did not change, as neither the soil pH nor the *nirS* abundance changed significantly as the grazing intensity increased. In general, the abiotic and biotic factors, NH_4^+-N and AOA abundance, were the dominant drivers for the promotion of PNA in soil. The soil pH, which showed an insignificant response to the grazing intensity, mainly determined the variations in the soil PDA in different treatments (Figure 7).

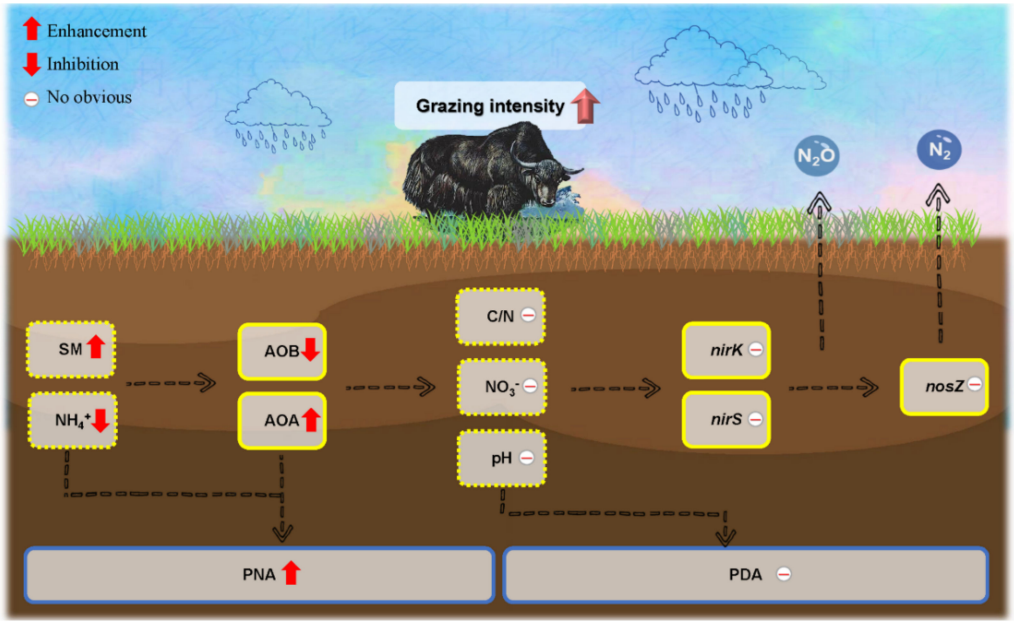

Figure 7. Changes in the soil PNA and PDA in response to different grazing intensities in an alpine meadow.

5. Conclusions

In this study, we found that the soil PNA was more responsive than PDA with the grazing intensity increase, and the variations in the PNA and PDA were determined by different factors. In contrast to our hypotheses, our results showed that the soil C availability was not the main control on the PNA and PDA. The changes in the AOA were more significant and positive than the changes in the AOB because of its low oxygen demand and its ability to adapt to the low NH_4^+-N concentration. The AOA contributed the highest proportion of the change in the PNA, followed by the soil NH_4^+-N availability. More than 90% of the variation in the PDA was explained by the pH, mainly by regulating the *nirS* abundances. Few researchers have studied how the abundances and potential activities of denitrifiers and nitrifiers vary at various grazing intensities in alpine meadow before. The results from this study can support the application of gene-based N-cycling models, as they can highlight the specific response patterns of nitrifiers and denitrifiers. Applications of such models could improve (1) our predictions of nitrification and denitrification activities at different grazing intensities in alpine meadow soil, and (2) our understanding of how grazing influences N_2O emissions on the Qinghai–Tibet Plateau. To better understand and predict the N-cycling model and N_2O emissions, research about other N-cycling processes and the corresponding microbial activities can be strengthened in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101521/s1, Figure S1 [35]: The grazing experiment in alpine meadow of the Qinghai-Tibet Plateau. (a) showed the vegetation condition of the grazing plot under different grazing intensities. (b) is the layout of the experimental site. The plots were divided into four grazing intensities. UG, un-grazed; LG, light grazing; MG, moderate grazing; HG, heavy grazing. Each grazing intensity consists of three replicates; Figure S2: The priori structural equation model (SEM) of the relationship between PNA (a), PDA (b) and its main influencing factors in alpine meadow. The priori SEM was established based on the known effects among the abiotic and biotic predictors, PNA and PDA; Table S1 [95–99]: Primer pairs and amplification conditions.

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