# Variation Characteristics of Soil Ammonia-Oxidizing and Denitrifying Microorganisms under Different Grazing Intensities: Postprint

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## Abstract

Soil nitrification and denitrification functional microorganisms play key roles in nitrogen availability, nitrate leaching, and nitrous oxide greenhouse gas emissions, and are of great significance in indicating the impacts of different grazing intensities on ecosystems and predicting the degradation status of grassland ecosystems. Using long-term experimental plots with different grazing intensities (light, moderate, and heavy) in the arid and semi-arid grasslands of Inner Mongolia as the research object, we investigated the responses of the abundance, community structure, and diversity of soil ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and denitrifying bacteria to different grazing intensities using quantitative PCR and terminal restriction fragment length polymorphism (T-RFLP) methods. The results showed that soil pH and ammonium nitrogen content ranged between 7.90-8.18 and 6.37-35.92 mg/kg, respectively. Moderate grazing treatment significantly increased soil pH (P=0.03), while ammonium nitrogen content was highest under heavy grazing treatment (P=0.02). Soil heterotrophic respiration under different grazing intensities was significantly reduced compared with the no-grazing treatment (P=0.02). The abundance ranges of soil AOA-amoA and AOB-amoA genes were (4.94-7.60)×10^9 copies per gram of dry soil and (0.68-3.75)×10^6 copies per gram of dry soil, respectively. Grazing treatment had no significant effect on AOA-amoA gene abundance, while moderate grazing treatment significantly reduced AOB-amoA gene abundance (P=0.04). The abundance of denitrifying microbial nosZ gene was lowest under light grazing treatment (P=0.03). Soil ammonium nitrogen content was the main factor affecting AOA-amoA and AOB-amoA gene abundances, while nosZ gene abundance was mainly influenced by denitrification substrate content and soil aeration conditions. Redundancy analysis indicated that changes in available nitrogen content caused by grazing

were the main factor leading to significant changes in the community structures of ammonia-oxidizing and denitrifying microorganisms.

## Full Text

# Responses of Soil Ammonia Oxidizers and Denitrifiers to Different Grazing Intensities

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### Abstract

Soil nitrifiers and denitrifiers play key roles in determining soil nitrogen (N) availability, nitrate leaching, and N O emissions, and thus could serve as indicators of grazing intensity effects on grassland ecosystems as well as grassland degradation. In this study, soil samples were collected from a long-term field experiment with different grazing intensities (low-level, middle-level, and high-level grazing) in arid and semi-arid grasslands of Inner Mongolia. We analyzed the responses of ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and denitrifiers in terms of abundance, community composition, and diversity at different grazing intensities using real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) approaches. The results showed that soil pH and ammonium content ranged from 7.90–8.18 and 6.37–35.92 mg/kg, respectively. Middle-level grazing significantly increased soil pH ($P = 0.03$), whereas soil ammonium content was highest in the high-level grazing treatment ($P = 0.02$). Soil heterotrophic respiration was markedly lower under all grazing intensities compared to the non-grazing treatment. AOA-amoA and AOB-amoA gene abundances ranged from (4.94–7.60) $\times$ 10 and (0.68–3.75) $\times$ 10 copies/g dry soil, respectively. AOA-amoA gene abundance showed no significant change in any treatment, whereas middle-level grazing strongly decreased AOB-amoA gene abundance ($P = 0.04$). The abundance of nosZ gene (coding for nitrous oxide reductase) was significantly decreased in the low-level grazing treatment ($P = 0.03$). The abundances of AOA and AOB were significantly influenced by ammonium content, whereas nosZ gene abundance was influenced by substrate content and soil aeration. Redundancy analysis showed that the variation in N

availability induced by grazing was the major factor influencing the community composition of ammonia oxidizers and denitrifiers.

**Keywords:** ammonia-oxidizing archaea; ammonia-oxidizing bacteria; arid and semi-arid grassland; potential nitrification rate; denitrifying enzyme activity; grazing

## Introduction

Grazing is one of the primary land-use practices in grassland ecosystems. Animal grazing, trampling, and excretion directly or indirectly affect grassland plant community composition. Previous studies have shown that grazing significantly reduces aboveground plant biomass, thereby affecting plant nitrogen uptake and decreasing ecosystem productivity. Different grazing intensities also cause significant changes in aboveground plant productivity and community composition. As plants are key components of ecosystem material cycling, changes in aboveground plant biomass, abundance, and diversity inevitably affect belowground ecosystem functions and element cycling processes.

Grazing can directly or indirectly influence soil microbial metabolism, growth, and nutrient transformation. With increasing grazing intensity, soil organic matter and organic nitrogen content decrease significantly, while heavy grazing reduces soil available nitrogen content, thereby affecting plant nitrogen uptake. Light grazing can increase soil total nitrogen content and promote soil nitrogen cycling. Although numerous studies have examined grazing effects on plant productivity, soil environment, and nitrogen cycling, the response mechanisms of soil microorganisms involved in nitrogen cycling to grazing intensity remain unclear.

Ammonia oxidation is the rate-limiting step of soil nitrification and a critical link in the nitrogen cycle. The product of ammonia oxidation, nitrate, is reduced through denitrification to a series of nitrogen oxide gases and N , leading to soil nitrogen loss and increased greenhouse gas emissions, which exacerbate global climate change. Soil nitrifying and denitrifying functional microorganisms play key roles in nitrogen availability, nitrate leaching, and nitrogen oxide gas emissions. Studies have shown that grazing significantly reduces the abundance of ammonia-oxidizing and denitrifying microbial functional genes in desert steppe, thereby affecting soil available nitrogen content and ecosystem nitrogen loss. Light grazing can significantly stimulate belowground microbial activity, while moderate and heavy grazing affect soil nitrogen pools by altering mineralization and nitrification processes. Understanding the effects of grazing on microbially mediated ammonia oxidation and denitrification processes and their feedback mechanisms is crucial for comprehending nitrogen cycling in grassland ecosystems and their responses to human activities and climate change, providing scientific basis for grassland ecosystem restoration and rational utilization.

The temperate grasslands of northern China serve as important ecological barriers, playing irreplaceable roles in water conservation, biodiversity maintenance, and livestock product provision. However, overgrazing has caused severe grassland degradation in recent decades. This study was conducted at the Siziwang Banner experimental base of the Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences to analyze the effects of grazing intensity on soil physicochemical properties and the abundance, community structure, and diversity of functional microorganisms such as ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and denitrifiers, aiming to explore the response mechanisms of soil microorganisms to grazing disturbance in grassland ecosystems.

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## 1. Study Site and Experimental Design

The experimental site was located at the Siziwang Banner experimental base of the Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences (41°47 17  N, 111°53 46  E). The area has a mean annual temperature of 3.4 °C and receives 110–250 mm of precipitation annually, representing a semi-arid grassland ecosystem. The vegetation belongs to the zonal vegetation of *Stipa breviflora* desert steppe, with the plant community dominated by *Stipa breviflora* and *Cleistogenes songorica*.

The grazing experiment was established in [year not specified] and included four treatments: control (no grazing), light grazing, moderate grazing, and heavy grazing. The experimental area was divided into blocks, with each block containing all four treatments in a completely randomized layout. Stocking rates were 0, 0.45, 0.91, and 1.36 sheep units per hectare for the control, light, moderate, and heavy grazing treatments, respectively. Local adult Mongolian castrated sheep were used, with a total grazing period of [duration not specified] days per year.

Soil samples were collected in August 2015. In each plot, five sampling points were randomly selected using a soil auger (5 cm diameter) to collect 0–10 cm depth soil cores. Samples from each plot were mixed to form a composite sample. Visible stones and plant residues were removed, and the soil was passed through a 2 mm sieve. One portion was stored at -80 °C for molecular analysis, while another portion was air-dried for physicochemical analysis.

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## 2. Soil Physicochemical Properties and Microbial Activity Measurements

Soil water content was determined by oven-drying fresh soil at 105 °C for 24 h. Soil pH was measured in a 1:2.5 (g/mL) soil-to-water suspension using a pH meter (Delta 320, Mettler-Toledo Instruments Co., Shanghai, China). Soil

organic carbon content was determined by the conventional potassium dichromate volumetric method. Ammonium nitrogen (NH -N) and nitrate nitrogen (NO -N) were extracted with 1 mol/L KCl (1:5, g/mL) and measured using a flow analyzer (SAN++, Skalar, Holland).

Potential nitrification rate and denitrifying enzyme activity were measured using the chlorate inhibition method and acetylene inhibition method, respectively, following procedures described in previous studies. Soil heterotrophic respiration, which reflects microbial decomposition of soil organic matter, was measured by incubating fresh soil at 25 °C for 24 h and analyzing CO concentration using a gas chromatograph (Agilent 7890A GC System).

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### 3. DNA Extraction and Real-Time Quantitative PCR

Soil DNA was extracted from 0.25 g of fresh soil using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., San Diego, CA, USA) following the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Samples were stored at –20 °C and diluted 10-fold for downstream molecular analyses.

Quantification of AOA-amoA, AOB-amoA, and nosZ genes was performed on an iCycler iQ5 system (Bio-Rad Laboratories, Inc., USA) using SYBR Green as the fluorescent marker. The 25 L reaction mixture contained 12.5 L 2× SYBR Premix Ex Taq (Takara Biotechnology, Japan), 0.5 L each of forward and reverse primers (10 mol/L), 2 L of diluted DNA template (1–10 ng), and sterile water to volume. Primer sequences and thermal conditions are listed in Table 1. Standard curves were constructed using serially diluted plasmids as described previously. Amplification efficiencies for all genes ranged from 85% to 98%, with $R^2 > 0.99$. Specificity was confirmed by melting curve analysis and agarose gel electrophoresis.

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### 4. Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis

T-RFLP analysis was used to assess the effects of grazing intensity on the community structure of AOA-amoA, AOB-amoA, and nosZ genes. The principle involves PCR amplification of target genes with fluorescently labeled primers, followed by restriction enzyme digestion to produce terminal restriction fragments (T-RFs) of varying lengths. The resulting T-RF patterns, with peaks representing different microbial taxa, were analyzed to compare community structure and diversity among treatments.

For T-RFLP analysis, the forward primers were labeled with 6-carboxyfluorescein (FAM). PCR amplification used the conditions specified in Table 1. Purified PCR products (500 ng) were digested with appropriate restriction enzymes:

HhaI (Promega, USA) for AOA-amoA, MspI (Takara Biotechnology, Japan) for AOB-amoA, and HhaI for nosZ genes. Digestion was performed in 20 L reactions with 4 U of enzyme. Digested products were sent to a sequencing company (TSINGKE, Beijing) and analyzed on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, USA). T-RFs differing by less than 1 bp were combined. Relative abundances were calculated as the peak area of each fragment divided by the total peak area, with fragments <1% relative abundance excluded from analysis.

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**5. Statistical Analysis**

Statistical analyses were performed using SPSS 19.0 and R 3.3.2. One-way ANOVA with Duncan' s post-hoc test was used to examine significant differences among treatments ($P < 0.05$). Spearman correlation analysis evaluated relationships between soil physicochemical properties, microbial activities, and functional gene abundances. Permutational multivariate analysis of variance (PerMANOVA) based on Bray-Curtis distances assessed effects of grazing intensity on microbial community structure and diversity. Shannon diversity index was calculated for each functional gene. Redundancy analysis (RDA) was used to reveal relationships between soil properties and microbial community structure.

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**Results**

**1. Effects of Different Grazing Intensities on Soil Physicochemical Properties and Microbial Activity** Soil pH ranged from 7.90 to 8.18, showing a significant increase under moderate grazing ($P = 0.03$). Soil ammonium content ranged from 6.37 to 35.92 mg/kg, with grazing significantly increasing ammonium content ($P = 0.02$), peaking under heavy grazing. Soil heterotrophic respiration was significantly lower in all grazing treatments compared to the control, ranging from 1.54 to 4.70 L CO g $^{1}$ d $^{1}$. No significant effects of grazing intensity were observed on potential nitrification rate or denitrifying enzyme activity.

Spearman correlation analysis revealed that soil heterotrophic respiration was significantly negatively correlated with soil water content ( = -0.66, $P = 0.02$) and ammonium content ( = -0.66, $P = 0.02$), which decreased with increasing grazing intensity.

**2. Effects of Different Grazing Intensities on Abundances of Ammonia-Oxidizing and Denitrifying Functional Genes** Real-time PCR quantification showed that AOA-amoA and AOB-amoA gene abundances ranged from (4.94–7.60) × 10 and (0.68–3.75) × 10 copies/g dry soil, respectively. Grazing intensity had no significant effect on AOA-amoA gene

abundance. However, moderate grazing significantly decreased AOB-amoA gene abundance (P = 0.04), reducing it by 57.6% compared to the control. The nosZ gene abundance ranged from (2.49–5.78) × 10  copies/g dry soil, with light grazing significantly decreasing its abundance (P = 0.03) by 18.1% compared to the control.

Correlation analysis indicated that AOA-amoA and AOB-amoA gene abundances were significantly negatively correlated with soil heterotrophic respiration ( = -0.69, P = 0.01) and ammonium content ( = -0.78, P < 0.01), respectively. The nosZ gene abundance was significantly positively correlated with ammonium content ( = 0.58, P = 0.04) and negatively correlated with soil water content ( = -0.60, P = 0.04).

**3. Effects of Different Grazing Intensities on Ammonia-Oxidizing and Denitrifying Community Structure** T-RFLP analysis revealed that grazing significantly affected the community structure of amoA genes. For AOA-amoA genes digested with HhaI, three dominant T-RFs were detected (541 bp, 265 bp, and 352 bp) with average relative abundances of 59.5%, 21.4%, and 9.3%, respectively. For AOB-amoA genes, three T-RFs (70 bp, 130 bp, and 154 bp) showed average relative abundances of 25.3%, 24.7%, and 22.2%, respectively. The nosZ gene yielded four main T-RFs (109 bp, 190 bp, 75 bp, and 204 bp) with relative abundances of 40.7%, 18.2%, 12.3%, and 10.8%, respectively.

Shannon diversity indices for AOA-amoA, AOB-amoA, and nosZ genes all showed significant decreasing trends with increasing grazing intensity (P < 0.05), with moderate and heavy grazing significantly lower than light grazing. Diversity indices were significantly positively correlated with soil water content and ammonium content (P < 0.05).

PerMANOVA analysis showed that grazing intensity had significant effects on the community structure of AOA-amoA (P = 0.04) and nosZ genes (P = 0.03), but not on AOB-amoA genes (P = 0.26).

Redundancy analysis (RDA) revealed that soil properties explained 40.1% and 10.4% of the variation in AOA-amoA and AOB-amoA community structures, respectively. Soil organic carbon (P = 0.03) and ammonium content (P = 0.02) were significant factors influencing AOA-amoA community variation. For nosZ genes, the first two axes explained 45.7% of community variation, with nitrate content being a significant influencing factor (P = 0.08).

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## Discussion

**1. Effects of Different Grazing Intensities on Soil Physicochemical Properties and Microbial Activity** Grazing effects on soil physicochemical properties vary with intensity. In this study, soil pH showed a small but significant increase under moderate grazing (P = 0.03), though the magnitude

of change (7.90–8.18) was insufficient to substantially impact soil functional microorganisms. Grazing significantly increased soil ammonium content, consistent with other studies, likely due to increased livestock excretion inputs with higher grazing intensity.

Soil heterotrophic respiration is an important indicator of microbial activity, reflecting the rate and intensity of soil organic matter decomposition. This study found that grazing significantly reduced soil heterotrophic respiration. Previous research at this site showed that soil carbon-to-nitrogen ratio decreased significantly with grazing intensity (range: 9.56–10.26), suggesting that reduced microbial carbon availability contributed to lower heterotrophic respiration.

**2. Effects of Different Grazing Intensities on Soil Ammonia-Oxidizing and Denitrifying Microorganisms** Ammonia-oxidizing and denitrifying microorganisms are key functional groups in soil nitrogen transformation, and their abundance and activity are closely related to ecosystem nitrogen cycling. While some studies have reported that grazing significantly increases amoA gene abundance, this study found no significant change in AOA-amoA gene abundance with grazing intensity, consistent with previous research. AOA may be less responsive to grazing than AOB in alkaline soils, being more influenced by soil physicochemical properties than by grazing disturbance.

Soil available substrate content is an important factor affecting ammonia-oxidizing microorganisms. With increasing grazing intensity, soil ammonium content increased significantly while nitrate showed no significant differences among treatments. The significant decrease in AOB-amoA abundance under moderate grazing may be attributed to sampling timing shortly after grazing events, when high ammonium accumulation could have inhibited AOB growth. Over time, as ammonium is gradually converted to nitrate, the abundance and diversity of ammonia-oxidizing microorganisms may stabilize.

RDA results showed that soil ammonium and nitrate contents significantly affected amoA gene community structure, indicating that increased available nitrogen from livestock excretion was a primary driver of community changes. The nosZ gene abundance was significantly positively correlated with ammonium content, as it participates in the final step of denitrification and is influenced by substrate availability, soil aeration, and other environmental conditions. Grazing reduced soil water content, which decreased denitrification substrate (nitrate) content, yet increased soil compaction under higher grazing intensity could promote denitrifier growth. The inconsistent results regarding nosZ abundance across studies may be attributed to differences in grazing intensity definitions, geographic location, and soil type.

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## Conclusion

This study demonstrated that: (1) Moderate grazing significantly increased soil pH (P = 0.03), while ammonium content was highest under heavy grazing (P = 0.02). Soil heterotrophic respiration was significantly reduced by all grazing intensities compared to the control (P = 0.02). (2) AOA-amoA and AOB-amoA gene abundances ranged from (4.94–7.60) × 10  and (0.68–3.75) × 10  copies/g dry soil, respectively. While grazing had no significant effect on AOA-amoA abundance, moderate grazing significantly decreased AOB-amoA abundance (P = 0.04). The nosZ gene abundance ranged from (2.49–5.78) × 10  copies/g dry soil, being significantly reduced under light grazing (P = 0.03). (3) Soil ammonium content was the primary factor influencing AOA-amoA and AOB-amoA abundances, while nosZ abundance was mainly affected by denitrification substrate content and soil aeration. Grazing significantly affected amoA gene community structure, and the diversity of AOA-amoA, AOB-amoA, and nosZ genes all decreased with increasing grazing intensity, with moderate and heavy grazing being significantly lower than light grazing. Changes in available nitrogen content induced by grazing were the main factor driving significant shifts in ammonia-oxidizing and denitrifying microbial communities.

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*Note: Figure translations are in progress. See original paper for figures.*