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# Effects of grazing on $N_2O$ production potential and abundance of nitrifying and denitrifying microbial communities in meadow-steppe grassland in northern China

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ABSTRACT

*Purpose:* The aim of this study was to investigate the effects of cattle grazing on the nitrous oxide ($N_2O$) production potential from meadow-steppe grassland soil in northern China, and the relationship between cattle grazing and the abundance of different functional microbial genes for potential of $N_2O$ emissions.
*Materials and methods:* We collected soil samples at a depth of 0–20 cm over six times during two plant growing seasons in 2011 and 2012 on a native *Leymus chinensis* grassland. At each of the six sampling occasions, soil samples were taken from three pairs of the cattle grazed vs. ungrazed plots. We then determined (1) the soil moisture, pH, total carbon and nitrogen, and mineral N ($NH_4^+$ and $NO_3^-$) content, (2) the potential rates of $N_2O$ production from nitrification ($N_{N_2O}$) and from denitrification ($D_{N_2O}$ and $D_{N_2}$) using the acetylene inhibition method, and (3) the abundance of the *amoA* (ammonia mono-oxygenase) gene of ammonia-oxidizing archaea (*AOA*) and ammonia-oxidizing bacteria (*AOB*), the *narG* (nitrate reductase) gene and *nosZ* (nitrous oxide reductase) gene using quantitative real-time PCR (qPCR). The relationship among the changes in the $N_2O$ production potential rates, the abundance of microbial functional groups and the soil environment was analyzed using mix effects modeling and structural equation modeling.
*Results and discussion:* The *AOA*, *AOB*, *narG*, *nosZ* genes and the potential $N_2O$ production rate all varied significantly with the season ($P < 0.01$). Grazing induced an overall reduction in soil moisture ($P < 0.05$) and soil total N in 2012 ($P < 0.05$), and a significant increase in the abundance of *AOB* genes ($P < 0.05$); but no significant difference between grazing treatments was found on the abundance of *AOA*, *narG* and *nosZ* genes, or on the $N_{N_2O}$ and $D_{N_2O}$.
Approximately 80% of the variation in $N_{N_2O}$ could be explained by the abundance of *AOA* and *AOB* genes ($P < 0.0001$), which in turn was explained by soil $NH_4^+$ content and soil moisture; The abundance of *narG* gene, along with total C, $NO_3^-$ content and soil moisture, explained 87% of variation in the $D_{N_2}$ ($P < 0.0001$). The abundance of *narG* gene was related to the production of N gases from denitrification ($D_{N_2O+N2}$), but not the $D_{N_2O}$. Soil moisture was the best predictor for $D_{N_2O}$.
*Conclusions:* The abundance of *amoA* and *narG* genes are good indicators for the potential nitrification and denitrification rates in the meadow steppe grassland. Soil moisture is the most important factor controlling the $N_2O$ emission potential in the meadow-steppe grassland. The grassland soils protected from animal grazing or that under a moderate grazing for five years did not show a significant difference in potential $N_2O$ emissions. Our results suggest that grazing induced grassland degradation may not necessarily be associated with a reduction in $N_2O$ emissions as reported in other semiarid grasslands in a more arid environment.


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## 1. Introduction

Nitrous oxide ($N_2O$) emissions contribute to global warming (IPCC, 2007) and to the catalytic depletion of the ozone layer (Ravishankara et al., 2009). $N_2O$ is mainly produced in soils by microbial nitrification which converts the ammonium ($NH_4$) to nitrite ($NO_2^-$), and then to nitrate ($NO_3^-$), and by microbial denitrification that reduces nitrogen oxides such as $NO_3^-$ to nitrogen gases ($N_2O$ and $N_2$) (Zumft, 1997). Temperate grasslands cover 11% of the earth's terrestrial surface (Sala et al., 2001), and are mostly used as grazing land for animal production. Animal grazing removes herbage which reduces vegetation cover and alters soil water and energy balance (Leriche et al., 2001), increases soil compaction or reduces soil aeration by tramping (Oenema et al., 2007; Houlbrooke et al., 2008), and changes the quantity and quality of soil organic matter and mineral N content by the deposition of dung and urine (Saggar et al., 2004). All of these effects have been shown to influence $N_2O$ emissions and $N_2O$ emissions from temperate grazed-grasslands are estimated to be more than 10% of the global budget (Oenema et al., 2007).

An increasing number of studies have been conducted in grasslands to quantify $N_2O$ production under different managements (Du et al., 2006; Groffman et al., 1993; Shan et al., 2011; Xu et al., 2008; Le Roux et al., 2007). These studies found that, for most of managed temperate grassland under humid climate, $N_2O$ was predominantly produced from denitrification (de Klein and Van Logtestijn, 1994; Wrage et al., 2001; Saggar et al., 2004, 2007a); and animal grazing would increase $N_2O$ emission by enhanced N cycling rate (e.g.; Hyde et al., 2006; Liebig et al., 2006; Luo et al., 2008). While for most of the arid and semiarid grasslands, the $N_2O$ was predominantly produced by microbial nitrification (Cookson et al., 2006; Verchot et al., 2002; Xu et al., 2008), animal grazing generally decreased $N_2O$ emissions by the grazing-induced reduction in soil organic matter and soil moisture (Phetteplace et al., 2001; Wang et al., 2005). These studies attribute the observed changes in $N_2O$ emissions to the changes in soil conditions and their effects on carbon (C) and N cycling, and had no explicit consideration of the role that microorganisms played in $N_2O$ production. This picture has changed recently, with increasingly more research to investigate the abundances of different microbial functional genes and their relationship with $N_2O$ production, as well as their changes under different grassland management (Di et al., 2009; Philippot et al., 2009). Le Roux et al. (2007) reported that grazing led to an increase in the abundance of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) genes and therefore the potential for nitrification in semi-natural grassland. Wakelin et al. (2009) also indicated that pasture management did not affect the abundance of *amoA* and *narG* gene. So far as the preceding literature, no consistent relationships were found between the abundance of functional microbial genes and the potential of nitrification and denitrification, nor between animal grazing effects and the abundance and activity of denitrifying microbes (functional genes for denitrification) and their activities (Attard et al., 2011; Chroakova et al., 2009; Philippot et al., 2009). Furthermore, most studies focused on one process of the N cycle, i.e., either on nitrification or on denitrification (Chroakova et al., 2009; Di et al., 2009; Philippot et al., 2009), and so was unable to completely describe the relations between the overall $N_2O$ production potential from grassland soils, the microbial functional groups involved, and their changes under grazing.

In this study we report the effects of animal grazing on $N_2O$ production processes from native *Leymus chinensis* meadow-steppe grassland on the Hulunber high plain in North Eastern China. The *L. chinensis* grassland covers an area of about 90,000 $km^2$ and is one of the most well-known rangelands in the east part of the Eurasian steppe (Wang, 2004). However, grazing-induced grassland degradation has profoundly affected the grassland ecosystems, and led to significant depletion in soil organic matter and biomass production (Li et al., 1997, 2008). The effects of grazing on this ecosystem have been increasingly studied in order to support sustainable land management for both animal production and environmental impacts. Previous studies cover the impact of animal grazing on plant species diversity and productivity (Zhou et al., 2006; Gao et al., 2012) and soil quality (Han et al., 2008). However, no information is available on $N_2O$ emissions and abundance of functional microbial genes.

The aim of the present study was to determine the impact of animal grazing on the $N_2O$ production potential over the plant growing season (May–September) and abundance of microbial functional genes related to $N_2O$ emissions in the *L. chinensis* meadow-steppe grassland soil. This grassland is under a semi-arid climate, though the rainfall in its distribution area is relatively higher than that of the typical steppe or desert steppe grasslands (Wang et al., 1985). We hypothesized that animal grazing would reduce the abundance and activities of nitrifying and denitrifying microbial genes and reduce the $N_2O$ production potential from this meadow-steppe grassland soil, and that the reduction would be associated with the grazing-induced decrease in soil moisture, plant production and soil C and N content in this semiarid environment.

## 2. Materials and methods

### 2.1. Experimental grassland

The experiment was conducted in the cattle grazing enclosure of the Hulunber Grassland Ecosystem Research Station of the Chinese Academy of Agricultural Sciences (49°22′N, 120°02′E, 600 m a.s.l.) in Inner Mongolia, China. The climate in this region is semi-arid. Annual mean precipitation is 400 mm with a large inter-annual variation from 150 to 550 mm, falling mainly during June–August which coincides with the plant growing season. Annual mean air temperature is 0 °C, with a monthly mean temperature of −26 °C in the coldest month (January) and 21 °C in the warmest month (July) (Chen et al., 2012). The region has a winter period of nearly six months, from October to March, and a short spring (April/May) and autumn (August/September). The frost-free period for plant growth spans about 105 days, from early May to early September (Wang, 2004).

The native meadow-steppe grassland is dominated by *L. chinensis*. Other prevalent grasses include *Stipa baicalensis*, *Cleistogenes squarrosa* and *Carex duriuscula*. The average annual grass production (net herbage accumulation) is about 2500 kg dry matter (DM) $ha^{-1}$, with a large variation of 1500–4000 kg DM $ha^{-1}$ (Chen et al., 2012). The soil is a dark chestnut soil (or Calcicorthic Aridisol in the US soil taxonomy classification system) with a pH of 7.1, and a soil bulk density of 1.27 g $cm^{-3}$.

The experimental grassland was on flat areas and enclosure was established in 2006 to investigate the effects of cattle grazing on grassland ecosystems, including multiple grazing intensities. The native grassland was historically the extensive rangeland of nomadic herders and was used with a very low stocking intensity. The stocking intensity on the grassland began to increase some four or five decades ago, and was assessed as being at a 'moderate to high' level at the time of setting up the experimental enclosure in 2006 (Wei et al., 2011). After the establishment of the enclosure, the grassland in the control paddock has remained ungrazed. That is, the grasses grow, senesce and litter naturally, with all plant material returned to soil (except a small fraction of the grasses consumed by native herbivores such as rodents and insects); while

**Table 1**
Enzymes encoded by functional genes measured in this study, and the thermal conditions and primer sequences used in qPCR.

| Functional gene | Enzyme | Annealing time and temperature | Elongation time and temperature | Primer | Primer sequence | Reference |
|---|---|---|---|---|---|---|
| *Bacterial amoA* | Ammonia monooxygenase | 55 °C, 30 s | 72 °C, 45 s | amoA1F, amoA2R | GGG GTT TCT ACT GGT GGT /CCC CTC KGS AAA GCC TTC TTC | Rotthauwe et al., 1997 |
| *Archaeal amoA* | Ammonia monooxygenase | 55 °C, 30 s | 72 °C, 45 s | CrenamoA23F, CrenamoA616R | ATGGTCTGGCTWAGACG /GCCATCCATCTGTATGTCCA | Francis et al., 2005 |
| *narG*[a] | Nitrate reductase | 58 °C, 30 s | 72 °C, 30 s | narGG-F, narGG-R | TCG CCS ATY CCG GCS ATGTC /GAG TTG TAC CAG TCR GCS GAY TCS G | Bru et al., 2007 |
| *nosZ*[b] | Nitrous oxide reductase | 60 °C, 30 s | 72 °C, 30 s | nosZ2F, nosZ2R | CGC RAC GGC AAS AAG GTS MSS GT /CAK RTG CAK SGC RTG GCA GAA | Henry et al., 2006 |

[a] Touch down starting at 63 °C temperature decrease of 1 °C per cycle for 6 cycles.
[b] Touch down starting at 65 °C temperature decrease of 1 °C per cycle for 6 cycles.

the grassland adjacent to the control paddock has been grazed at a moderate stocking intensity (about 0.34 cattle $ha^{-1}$) during the plant growing season from May to October (Wei et al., 2011). Our study compares the soils from the fenced control area that had been ungrazed for 5–6 years and that from the area grazed with moderate stocking intensity. By 2011 when soil sampling commenced, the grasslands showed a visible difference between the two areas, with much taller grass and standing dead and more little accumulation on soil surface in the non-grazed than in the grazed area.

### 2.2. Soil sampling and vegetation description

Soil samples were collected three times in each of two years: in spring (27 May), summer (27 July) and in autumn (27 September) in both 2011 and 2012. Three pairs of soil sampling plots were determined and marked as permanent soil sampling areas for repeated sampling and measurements on the both sides of the fence that divided the grassland into ungrazed (control paddock) and moderately grazed areas. Each sampling plot was approximately 5 × 5 $m^2$ and 10 m distant from the fence to avoid edge effects. The sampling in each time includes collecting three pairs of soil samples from the three grazed and three non-grazed plots. Soil was sampled at a depth of 20 cm, using a 7 cm diameter steel soil core. Five soil cores were randomly collected within each sampling plot and bulked together as a plot sample. Soil samples were then passed through a 2 mm sieve and stored at 4 °C in the laboratory until further use. Sub samples of fresh soil were stored at −20 °C for DNA extraction. Soil bulk density was determined once in July 2011 by sampling 50 $cm^3$ soils from the top 2.5 cm of the mineral soil. Standard methods of sample handling and laboratory analysis were followed as prescribed by Klute (1986).

The grassland vegetation was observed and measured once at the same time of soil sampling in summer (27 July) each year. Plant height was measured, and plant standing biomass was harvested, oven-dried at 65 °C for 48 h, and weighed for each species, using six quadrats of 1 $m^2$, one in each sampling plot (Li et al., 2008).

### 2.3. Chemical and microbial functional gene analyses

Soil $NH_4^+$ and $NO_3^-$ concentrations were determined in 2 M KCl extracts using a LACHAT Quickchem Automated Ion Analyzer (FIA Star 5010 Analyzer; Tecator). Gravimetric soil moisture content was determined by oven-drying at 105 °C for 24 h. Total soil C content was analyzed using the $H_2SO_4$–$K_2Cr_2O_7$ oxidation method (Nelson et al. 1996). Total N content was analyzed using Kjeldahl acid-digestion method with an auto-analyzer (Foss Inc., Hillerød, Sweden). The DNA was extracted from 0.3 g of frozen soil using MoBio Powersoil™ DNA Isolation Kit (San Diego, CA, USA) following the manufactures instructions and stored at −80 °C until further use. The abundance of ammonia monooxygenase A gene of ammonia-oxidizing archaea (*amoA-AOA*), ammonia-oxidizing bacteria (*amoA-AOB*), bacterial *narG* and *nosZ* genes were quantified in triplicate by real-time PCR using an iCycler IQ (Biorad). The real time PCR mixture contained 2 ng of undiluted soil DNA, 5 pmol of primers (Table 1) and 2× SYBR Green iCycler iQ mixture (Bio-Rad, US) in a total of 25 ml reaction volume.

### 2.4. Incubation experiment to measure $N_2O$ emission potential from nitrification and denitrification

The incubation experiment was performed in a 250 ml flask with 40 g (dry weight) of sieved field moist soil. The headspace inside the flask was set with three acetylene ($C_2H_2$) partial pressures: 0, 10 Pa and 10 kPa, each with three replicates (Yoshinari, 1993). Each flask was sealed with an airtight rubber lid and incubated at similar temperatures and moisture levels as recorded in the field. Gas samples of 1 ml from the headspace of the flasks were taken at 0, 1 and 7 days, and analyzed for $N_2O$ concentration using a gas chromatograph (Agilent 7890 GC USA) equipped with a $^{63}$Ni-electron capture detector operating at column.

The $N_2O$ production from nitrification was estimated by the headspace $N_2O$ concentration difference between flasks without $C_2H_2$ and that with 10 Pa $C_2H_2$. The $N_2O$ evolved by denitrification was estimated by the headspace $N_2O$ concentration in the flasks with $C_2H_2$ at 10 Pa. The $N_2$ evolved by denitrification was estimated by the headspace $N_2O$ concentration difference between the flasks with $C_2H_2$ at 10 kPa and with $C_2H_2$ at 10 Pa. The 10 kPa $C_2H_2$ concentration inhibits the reduction of $N_2O$ to $N_2$ (Klemedtsson et al., 1988; Webster and Hopkins, 1996).

### 2.5. Statistical analysis

The statistical analysis employed a repeated measure linear mixed effects model using the packages 'nlm' and 'predictmeans' in R package version 3.1 (Pinheiro et al., 2007). Data were analyzed using a model where the sampling plot was considered as a random effect and the grazing treatment, sampling time (combination of year and month) and their interaction as fixed effects. The correlation structure within each PLOT along the time was compound symmetry. The data for abundance of amoA-*AOB* and the $N_2O$ production potential were log transformed to satisfy the requirement for normality.

Structural equation modeling (SEM) was performed using Amos 20® (Amos Development Corporation, Crawfordville, FL, USA) with all the data to explore the causal links between soil physical, chemical and biological variables and $N_2O$ production potentials. The model considered soil moisture (SM), soil temperature (ST), total C, total N, $NH_4^+$, $NO_3^-$, the abundance of *amoA-AOA*, *amoA-AOB*, *narG* and *nosZ* genes and $N_2O$ production potentials from nitrification ($N_{N_2O}$), denitrification ($D_{N_2O}$) and $N_2$ production potential

($D_{N_2}$). Since SEM was most appropriate for datasets with large sample sizes (McCune et al., 2002) and the sample size ($n = 36$) in our experiment was low relative to the number of variables in this modeling, we also did another SEM for a better fit of the model to the data by aggregating all the soil parameters (SM, ST, C and N) into one variable "soil conditions" using principal component analysis (SPSS 14.0, Grace and Jutila, 1999). Small sample sizes generally result in conservative fit estimates (Shipley, 2000). In SEM, a $\chi^2$ test is used to determine whether the covariance structures implied by the model adequately fit the actual covariance structures of the data. A non-significant $\chi^2$ test ($P > 0.05$) indicates adequate model fit. The coefficients of each path as the calculated standardized coefficients were determined using the analysis of correlation matrices. Paths in this model were considered significant with a $P$-value <0.05. These coefficients indicate by how many standard deviations the effect variable would change if the causal variable was changed by one standard deviation (Cantarel et al., 2012; Petersen et al., 2012).

## 3. Results

### 3.1. Environmental conditions

Annual precipitation in 2011 (178 mm) and 2012 (210 mm) was both lower than the long term average (400 mm) at the experimental site. Monthly precipitation in May and July was higher in 2011 than 2012, but that in September was lower in 2011 than 2012 (Fig. 1). Average monthly air temperature was similar between the two years, ranging from −30 (January) to 20 °C (July). The average air temperature (Fig. 1) and soil temperature at 10 cm at the sampling months were both slightly higher in 2012 than 2011. The average soil temperature in May, July and September was 11, 20 and 10 °C in 2011, and 12, 21 and 12 °C in 2012.

The average plant standing biomass measured at the time of plant peak biomass (27th July) was 2880 and 2680 kg DM $ha^{-1}$, respectively in 2011 and 2012 in the un-grazed plots, and 470 and 320 kg DM $ha^{-1}$, respectively in 2011 and 2012 in the grazed plots.

### 3.2. Soil analysis

The soil moisture content varied from 7% to 34% (Fig. 2A) during the two year experimental period and showed significant interaction with time and grazing treatment ($P = 0.03$). Soil moisture content was significantly lower for the grazed treatment in May 2011 ($P = 0.0002$), July 2011 ($P = 0.006$), May 2012 ($P = 0.0002$) and in September 2012 ($P = 0.005$).

The total carbon (TC) content of soil was significantly lower ($P = 0.01$) in the grazed than un-grazed treatment (33.15 and 36.90 g $kg^{-1}$ respectively), and varied significantly with time ($P < 0.0001$) (Fig. 2B), but no interaction was observed between grazing treatment and time. In contrast, there was a strong interaction between grazing treatment and time for soil total nitrogen (TN) ($P = 0.0001$). Similar to TC, TN was also lower in the grazed treatment but this difference was only significant in the 2012 (Fig. 2C).

The $NH_4^+$ content was significantly lower ($P = 0.0001$) in grazed than ungrazed soils at May 2011 but was same for both treatments at other sampling times. The $NO_3^-$ content was significantly higher ($P = 0.0003$) in grazed than ungrazed soils at July 2011, but was significantly lower ($P = 0.0074$) in grazed than ungrazed soils at May 2012 (Fig. 2C and D).

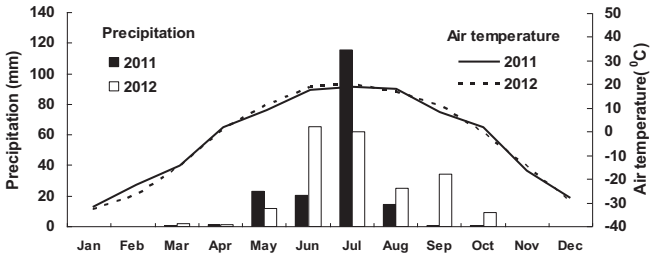


**Fig. 1.** Monthly total precipitation and mean air temperature at the experimental site during 2011 and 2012. This dataset is provided by Hulunber Grassland Ecosystem Research Station, the Chinese Academy of Agricultural Sciences (CAAS).

### 3.3. Microbial functional genes

The *amoA* gene abundance of *AOA* and *AOB* in both grazing treatments varied significantly with time ($P < 0.01$ for grazing treatment × time interaction). The *AOA-amoA* gene abundance of grazed soil was significantly higher than ungrazed soils at May 2011 but was similar for both treatments at other sampling times. The *AOA*-amoA gene abundance was significantly higher ($P < 0.05$) in 2011 soil samples than in samples in 2012 (Fig. 3A). The *AOB*-amoA gene abundance of grazed soil was significantly higher than ungrazed soils at July 2011 ($P = 0.05$) and September 2011 ($P = 0.001$) but there was no significant difference between for grazing treatments at other sampling times (Fig. 3B). The *AOB-amoA* gene abundance in the grazed soils was significantly ($P < 0.05$) higher in 2011 than in 2012. The *AOB*-amoA gene abundance in ungrazed soils measured in 2011 was also significantly higher ($P < 0.05$) at May and July, but lower ($P < 0.05$) at September, than in the grazed soils.

The grazing treatment did not significantly influence the *narG* and *nosZ* gene abundance. However, the *narG* gene abundance varied with time ($P < 0.001$) (Fig. 3C).

### 3.4. $N_2O$ production potential from nitrification and denitrification

There was no significant effect of grazing treatment on total potential $N_2O$ production rate ($N_{N_2O} + D_{N_2O}$) or on the potential $N_2O$ production rate from nitrification ($N_{N_2O}$) or from denitrification ($D_{N_2O}$), respectively (Fig. 4A–C). The potential $N_2$ production rate ($D_{N_2}$) due to denitrification was significantly higher in ungrazed than grazed soils (Fig. 4D). These potential $N_2O$ production rates varied significantly with time ($P < 0.0001$) (Fig. 4A–D).

The contribution of $N_{N_2O}$ to total potential $N_2O$ production rate varied from 47.3% to 63.2%. In both years, the $N_{N_2O}$ was higher than $D_{N_2O}$ in May and July, but lower in September (Table 2).

### 3.5. Correlations between the abundance of microbial functional genes and potential $N_2O$ production rates

The potential of $N_2O$ production from nitrification ($N_{N_2O}$) showed a significant positive correlation with the abundance of *AOA-amoA* gene ($R^2 = 0.4653$; $P < 0.001$) or with the abundance of *AOB-amoA* gene ($R^2 = 0.4047$; $P < 0.001$) (Fig. 5A and B). The potential of $N_2O$ and $N_2$ production from denitrification ($D_{N_2O} + D_{N_2}$) showed a significant positive correlation with the *narG* gene abundance ($P < 0.001$) (Fig. 5C). No significant correlation was detected between $D_{N_2}$ and the abundance of *nosZ* gene.

### 3.6. Factors controlling nitrification and denitrification processes and nitrifying and denitrifying microbial communities in the soils

Path analyses indicated that the conceptual models for $N_{N_2O}$, and for $D_{N_2O}$ and $D_{N_2}$ (Fig. 6A and C) fit the observed data ($N_{N_2O}$ full model $\chi^2 = 5.886$, d.f. = 5, $P = 0.319$; $D_{N_2}$ full model $\chi^2 = 17.591$,

d.f. = 10, $P = 0.130$). After we removed non-significant paths and tested model fit to observed data again, the simplified final models also fitted well to the data ($N_{N_2O}$: $\chi^2 = 12.084$, d.f. = 9, $P = 0.209$; $D_{N_2}$: $\chi^2 = 12.501$, d.f. = 8, $P = 0.109$) (Fig. 6B and D). The first principal component of a PCA on all the four soil variables (SM, ST, TC and TN) explained 68.2% of the total variances, suggesting it a good descriptor of "soil conditions". Using this aggregated variable "soil conditions" to replace the SM, ST, TC and TN in the conceptual models (Fig. 6A and C), the simplified final models also fitted well to the data ($N_{N_2O}$: $\chi^2 = 5.16$, d.f. = 3, $P = 0.160$; $D_{N_2}$: $\chi^2 = 15.79$, d.f. = 12, $P = 0.201$) (Fig. 6E and F).

The final model with all soil variables separately modeled (in Fig. 6C) explained 79% of variation in the $N_{N_2O}$ ($P < 0.0001$), and the final model with soil variables aggregated as "soil conditions" (Fig. 6E) explained 55% of variation in the $N_{N_2O}$ ($P < 0.0001$). In both final models, the abundance of *AOA-amoA* and *AOB-amoA* genes was the direct explaining factors (Fig. 6C and E). The abundances of *AOA-amoA* and *AOB-amoA* genes were explained by the $NH_4^+$ content, and, for *AOB-amoA* genes only, also by soil moisture. The abundance of *AOA-amoA* was the most important controlling factor for $N_{N_2O}$ followed by the abundance of *AOB-amoA* (Fig. 6C and E), same as indicated by Fig. 5. Soil moisture had significant correlations with most of variables and influenced $N_{N_2O}$ via its effects on soil total C, total N, $NH_4^+$ and abundance of amoA genes (Fig. 6C).

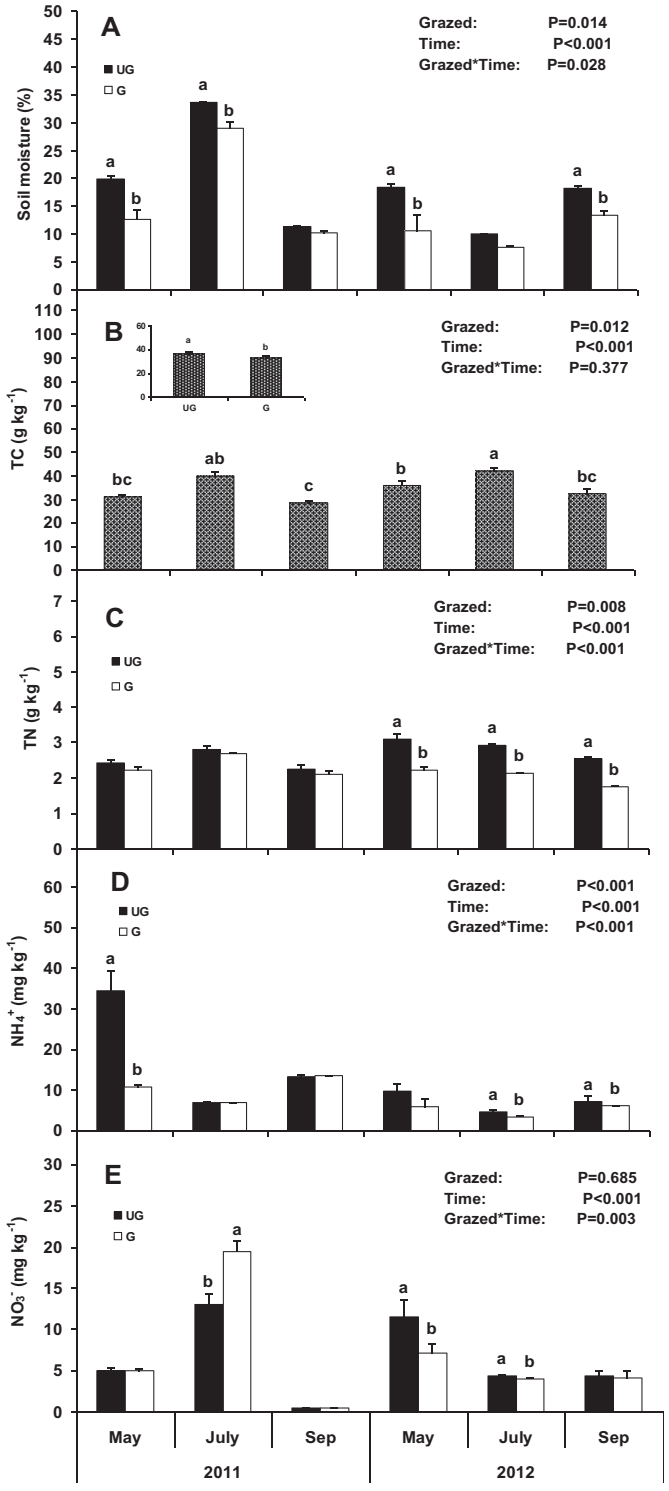


**Fig. 2.** Gravimetric soil moisture content (A), total carbon (TC) (B), total nitrogen (TN) (C), $NH_4^+$ (D) and $NO_3^-$ (E) content under in the *Lyemus chinensis* grassland. Treatment means ungrazed (UG■) and grazed (G□); time means different sampling month in 2011–2012. Bars are means ± 1 sem. Values followed by a different letter are significantly different within each sampling date ($P < 0.05$). No significant grazing × time interaction was detected for TC, so the dashed bars (▒) in (B) represent the mean TC at different time, with insets showing the difference grazed and ungrazed soils.

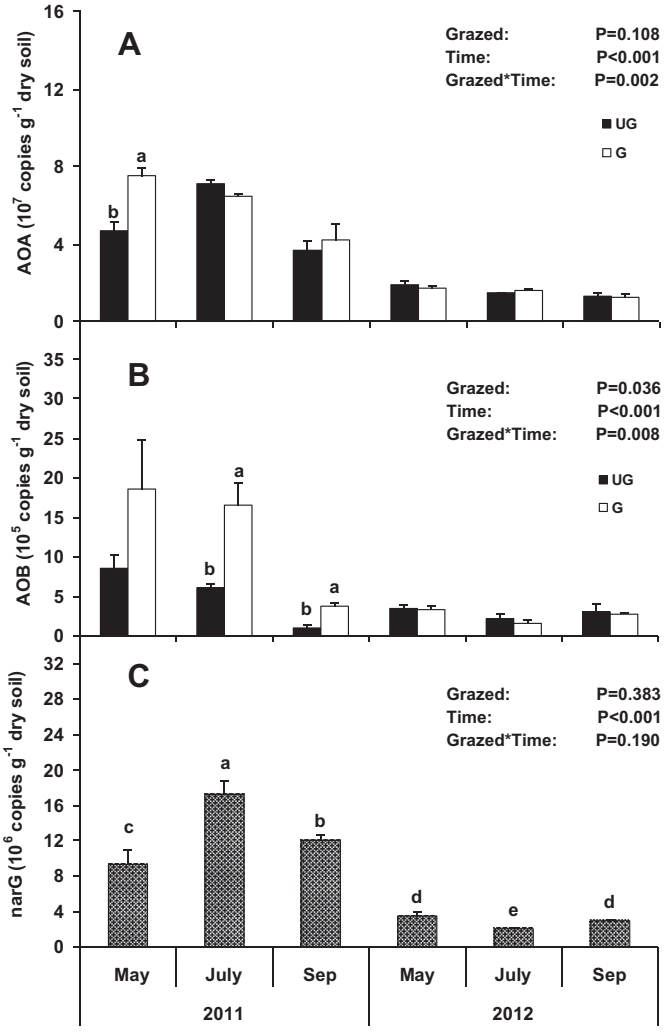


**Fig. 3.** Soil *AOA* gene (A), *AOB* gene (B) and *narG* gene (C) copy numbers in grassland soils. Treatment means ungrazed (UG■) and grazed (G□). Time means different sampling month in 2011–2012. Bars are means ± 1 sem. Values followed by a different letter are significantly different within each sampling date ($P < 0.05$). No significant grazing effects or grazing × time interaction effects were detected for *narG* gene, so the bars (▒) in (C) represent the mean values for *narG* in different time in both grazed and ungrazed soils.

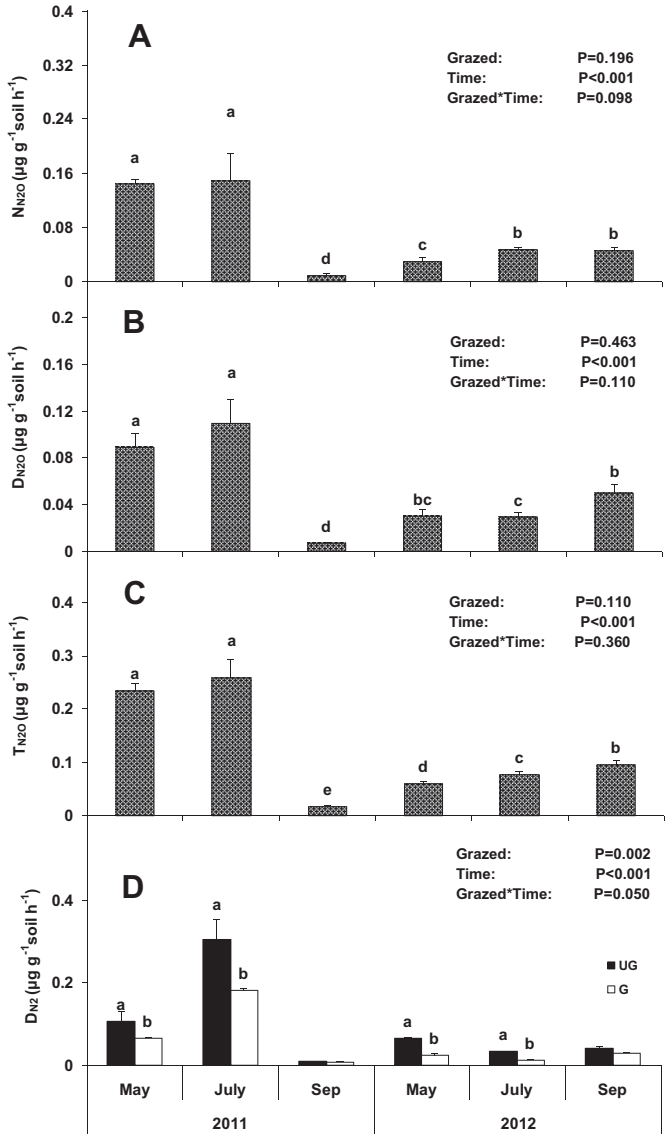


**Fig. 4.** The production potential of $N_{N_2O}$ (A), $D_{N_2O}$ (B), $T_{N_2O}$ (C) and $D_{N_2}$ (D) in grassland soils. Treatment means ungrazed (UG■) and grazed (G□). Time means different sampling month in 2011–2012. Bars are means ± 1 sem. Values followed by a different letter are significantly different within each sampling date ($P < 0.05$). No significant grazing effects or grazing × time interaction effects were detected for $N_{N_2O}$ (A), $D_{N_2O}$ (B) and $T_{N_2O}$ (C), so the bars (▯) in (A–C) represent the mean values for these values in different time in both grazed and ungrazed soils.

The abundance of *narG*, total C, $NO_3^-$ content and soil moisture explained 87% of variation in the $D_{N_2}$ ($P < 0.0001$) in Fig. 6D and 76.24% of variation in the $D_{N_2}$ ($P < 0.0001$) in Fig. 6F. Soil moisture is the most important factor affecting $D_{N_2O}$ and $D_{N_2}$ directly and affecting $D_{N_2}$ indirectly via affecting soil total C and $NO_3^-$ (Fig. 6D). Soil moisture was the only factor that explained the variation in $D_{N_2O}$. Interestingly, abundance of *nosZ* genes had no effect on $D_{N_2}$ ($P > 0.05$).

**Table 2**
The contribution of nitrification ($N_{N_2O}$) and denitrification ($D_{N_2O}$) to total $N_2O$ production.

| % | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | May | Jul | Sep | May | Jul | Sep |
| $N_{N_2O}$ | 62.3 ± 3.4 | 55.1 ± 9.7 | 47.3 ± 7.9 | 50.8 ± 1.6 | 63.2 ± 7.4 | 47.4 ± 5.8 |
| $D_{N_2O}$ | 37.7 ± 3.4 | 44.9 ± 9.7 | 52.7 ± 7.9 | 49.2 ± 1.6 | 36.8 ± 7.4 | 52.6 ± 5.8 |

## 4. Discussion

### 4.1. Grazing effects on the potential $N_2O$ production rate in meadow-steppe grassland

Many studies have shown that grazing affects soil nitrification and denitrification through affecting the abundance of microbial functional genes (Chroakova et al., 2009; Di et al., 2009, 2010); while in our study, there were no significant grazing effects on the abundance of microbial functional genes or on the potential $N_2O$ production rates over the two-year experimental period (Figs. 3 and 4). This result is different from that obtained in most studies on grazed pastures, such as that in New Zealand (Menneer et al., 2005) and Europe (Chroakova et al., 2009). Denitrification is the dominant process of $N_2O$ production from these grazed pastures

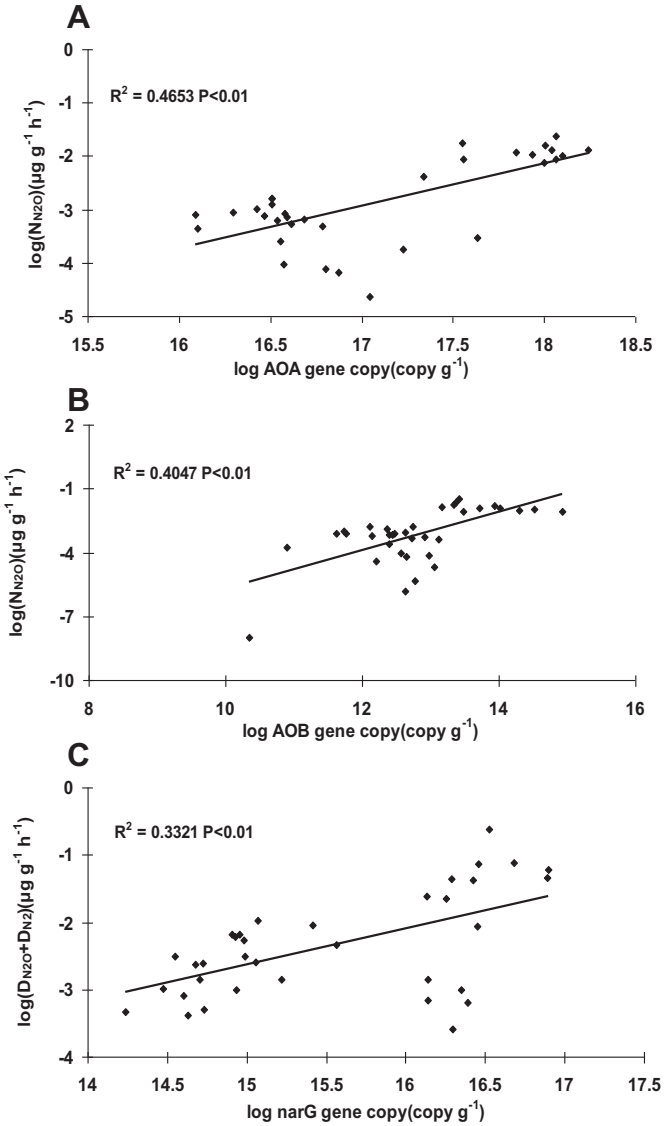


**Fig. 5.** Relationships between the abundance of *AOA* (A), *AOB* (B) genes and potential $N_2O$-production rates from nitrification; the abundance of *narG* (C) gene and the potential denitrification rate ($D_{N_2O} + D_{N_2}$) in grassland soils. All data were log-transformed before performing regression analysis. $R^2$ are the coefficients of determination.

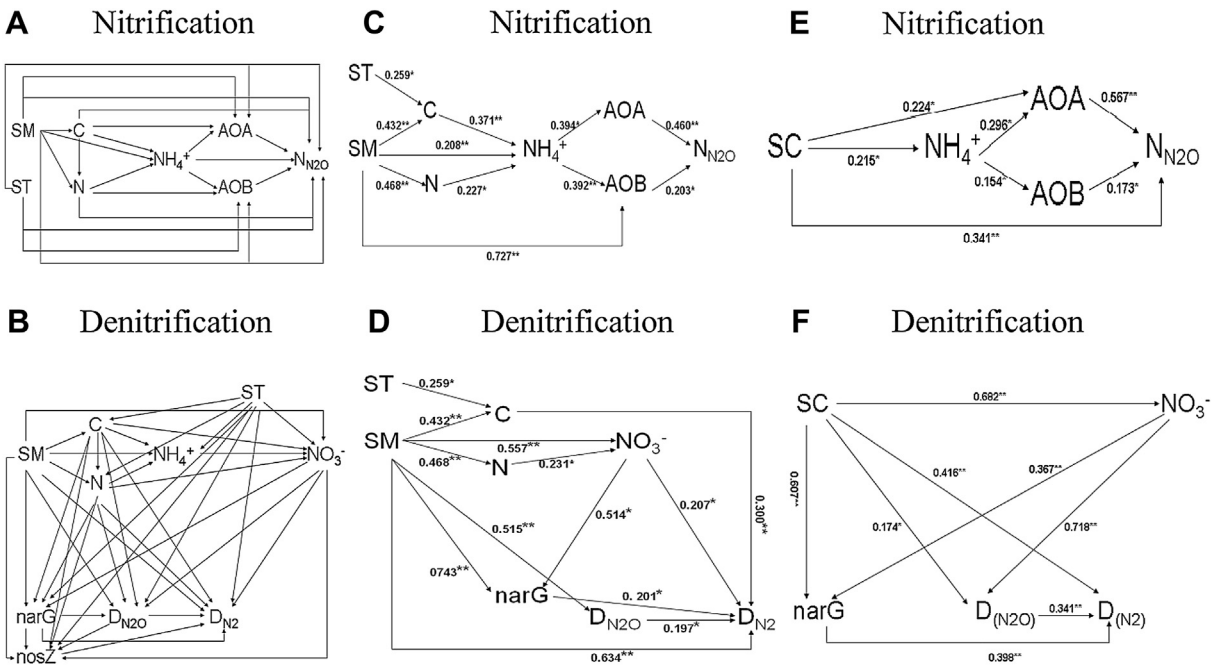


**Fig. 6.** Path diagrams representing the full models (A, B) and the final models (C, D, E, F) to describe the patterns observed in nitrification (A, C, E) and denitrification (B, D, F) rates. The full models A and B include all relevant variables like electron donors and electron acceptors; the final models C and D were simplified from the full models with all the soil variables explicitly modeled; the final models E and F were simplified from the full models (A and B) after the soil variables were aggregated as one variable of "soil conditions (SC)" using the first component of a PCA on soil moisture, temperature, total C and total N. Numbers associated with single headed arrows are partial regression coefficients of multiple regressions. SM – soil moisture; C – total carbon; N – total nitrogen; $N_{N_2O}$ – $N_2O$ fluxes in nitrification; $D_{N_2O}$ – $N_2O$ fluxes in denitrification; $D_{N_2}$ – $N_2$ fluxes in denitrification. The number on all the pathways are the coefficients, indicating the level of determination of the donor factor to acceptor factor, with significant levels denoted ($^*P < 0.05$ and $^{**}P < 0.01$).

under humid climate conditions (de Klein and Van Logtestijn, 1994; Wrage et al., 2001); the grazing enhancement of $N_2O$ production on these pastures is primarily associated with the enhancement of N and C cycling through animal excreta deposition and with the anaerobic conditions created by animal treading (Saggar et al., 2004, 2007a,b; Oenema et al., 2007; Keil et al., 2011). Our result is also different from those obtained from the semiarid grasslands where $N_2O$ is predominantly produced via nitrification, and where grazing-reduced $N_2O$ emissions have been reported to be related to the reduction in soil moisture (Phetteplace et al., 2001; Wang et al., 2006; Xu et al., 2008). Wang et al. (2006) reported a reduction in $N_2O$ emission from semiarid typical-steppe grassland in Inner Mongolia, and related the reduction with the observed lower soil moisture, $NO_3^-$ and organic N content under grazing. The soil moisture observed in the meadow-steppe grassland in this study (Fig. 2A) is much lower than that in moist temperate pastures reported by Hyde et al. (2006) and Luo et al. (2008), but is higher than that in most of the semiarid grasslands, such as that reported by Phetteplace et al. (2001) and Xu et al. (2008). Our measurements show a grazing-induced reduction in soil moisture, and soil total C and N content (Fig. 2A), but both the abundance of nitrifying and denitrifying microbial genes and the $N_{N_2O}$ and $D_{N_2O}$ showed no significant difference between the grazed and ungrazed soils.

This lack of significant difference between grazing treatments might be simply due to the fact that grazing induced changes in soil substrate (soil labile nitrogen and carbon) and environment were not great enough to lead to a detectable difference in the abundance and activities of functional microbes. The compensation between the positive effects of grazing on $N_{N_2O} + D_{N_2O}$ through stimulating N cycling rate and the negative effects through reducing soil moisture in this semiarid environment may be attributable to the observed insignificant difference between grazed and un-grazed soils in the abundance and activities of microbial groups. The greater litter accumulation, higher soil $NH_4^+$ content but lower *AOB* gene abundance in ungrazed than grazed soils, indicate a slow N cycling rate in ungrazed than grazed soils. The interaction between soil bulk density and soil moisture may also attributable to the insignificant difference in the abundance and activities of functional microbes. The soil bulk density is higher in grazed (1.27 g $cm^{-3}$) than un-grazed plots (1.13 g $cm^{-3}$), thus a significantly lower volumetric soil moisture content in grazed than in ungrazed soils does not necessarily mean a better aeration conditions; in fact, the water-filled pore space (WFPS) was not significantly different ($P = 0.247$) between grazed (17.7%) and ungrazed (21.0%) soils, though volumetric soil moisture content showed significant difference (Fig. 2A). That is, similar aeration conditions in the grazed and ungrazed soils may reduce the effects of the difference described in volumetric soil moisture content, and attribute to the observed similarity in the abundance of microbial functional genes and their activities (Figs. 3 and 4). Furthermore, during the two experimental years (2011 and 2012), the study area received a much lower precipitation compared to the long-term average (Fig. 1), so that soil moisture content was more similar to those in the soil of a typical steppe which is distributed in a region dryer than that of meadow steppe on a climatic gradient in Inner Mongolia. If the data measured from the soil samples at July 2011, the extremely wet soil following continuous rainy days (Fig. 1), were excluded, grazing would appear to decrease the potential $N_2O$ production rates and the abundance of *AOA*, *AOB* and *narG* genes (data not shown). Significantly lower $N_2O$ emission or $N_2O$ production potential rates were reported from the typical grazed steppe grassland soils (Wang et al., 2006; Xu et al., 2008).

This suggests that the grazed soil may have less $N_2O$ production potential than ungrazed due to the reduction in soil moisture; but

under wet conditions, grazing induced soil moisture reduction may improve the soil aeration and stimulate the $N_2O$ from nitrification ($N_{N_2O}$), which might be a larger influence than the inhibition to the $N_2O$ production from denitrification ($D_{N_2O}$).

### 4.2. Dynamics of the abundance of microbial functional groups and $N_2O$ production potential

The potential of $N_2O$ from nitrification and denitrification in grassland soil in the study is much lower than those from most of other grassland soils reported (e.g., Luo et al., 1999; Rudaz et al., 1999). The contribution of nitrification to total $N_2O$ production potential ($N_{N_2O}$%) is slightly higher than that from denitrification in the studied grassland, being 47–63% in the six measurements. This contribution is lower than that reported in the typical steppe grassland (64–88%) in Inner Mongolia (Ri et al., 2003), and similar to that in the alpine meadow grassland (annual average 53%) on Tibetan plateau (Du et al., 2011). This change in $N_{N_2O}$% is most likely related with the effect of soil moisture: $N_{N_2O}$% decreases with soil moisture increase. Soil moisture in studied meadow-steppe grassland is higher than that of typical steppe (Xu et al., 2008) but lower than that of alpine meadow (Rui et al., 2011).

The abundance of nitrifying and denitrifying functional genes and their activities varied significantly across the seasons. The abundance of *AOA*, *AOB* and *narG* genes and the $N_2O$ production potential was much higher in May and July in 2011 than in other sampling time (Figs. 3 and 4); this pattern is also shown by the soil moisture (Fig. 2A). However, the abundance of *AOA* and *narG* genes was very high in September 2011 (Fig. 3A and C), but the $N_{N_2O}$ and $D_{N_2O}$ were lower (Fig. 4), than in other sampling time, which is most likely associated with the lower soil moisture and air temperature in the month (Figs. 1 and 2A). Our results support the results of Petersen et al. (2012) that the abundance of microbial functional genes does not respond quickly to environment change.

### 4.3. The predictive power of the abundance of functional microbial groups to $N_2O$ production potentials

Recent studies on the relationship between the abundance of functional microbial groups and nitrification and denitrification rates have not showed a consistent trend. Our results indicated that the abundances of microbial *amoA* (*AOA* and *AOB*) and *narG* genes are good indicators for the $N_2O$ production potential through nitrification and denitrification on the studied grassland soils (Figs. 5 and 6). However these results are different from many other recent studies that suggest no correlation between these two parts (Ma et al., 2008; Miller et al., 2008; Baudoin et al., 2009; Djigal et al., 2010). One common feature in these studies was that they sampled soil only at one time and used one incubation experiment (Ma et al., 2008; Miller et al., 2008; Song et al., 2010), or the experimental field was a cultivated land with large environment change and disturbance (Baudoin et al., 2009; Attard et al., 2011). Our results are in agreement with those reported from other grassland ecosystems (Čuhel et al., 2010; Di et al., 2010; Chroakova et al., 2009) in which soil was not strongly interfered with by human activities.

The positive correlation between the abundance of functional microbial genes and the $N_2O$ production potential in our study is from repeated soil sampling, over two plant growing seasons, from the soils either under protection from grazing or under sustained grazing with a moderate stocking intensity. Our data and that of Di et al. (2010) and Chroakova et al. (2009) suggest that the changes in the abundance of functional microbial groups and in the $N_2O$ production potential are correlated across seasons. Our result is not in conflict with that of Miller et al. (2008) and Song et al. (2010) who reported no significant correlation between the abundance of functional microbial groups and $N_2O$ production potentials. When we excluded the effects of sampling time from our dataset, the correlation between $N_{N_2O}$ and the abundance of *amoA* gene or between $D_{N_2O+N_2}$ and the abundance of *narG* gene in each sampling were not significant ($P > 0.05$). That is, our data shows that the changes in the abundance of functional microbial groups and in the $N_2O$ production potential are synchronized across seasons, but the soil difference induced by animal grazing, was not big enough to induce a significant and consistent difference in the abundance of microbial functional genes and in the $N_{N_2O}$ or $D_{N_2O+N_2}$.

Gene copy numbers will not likely provide information on real-time process rates since such rates are dependent on environmental conditions. Fluctuations in environmental conditions can cause rapid changes in real-time process rates, but not necessarily affect gene abundance. Our results suggest that the abundance of microbial functional genes are likely to be robust indicators for predicting effects on process rates of the long-term environmental changes in grassland ecosystem.

We found no obvious relationship between the abundance of *nosZ* genes and $N_2$ production potential ($R^2 = 0.0006$, $P > 0.05$); and soil moisture, total C and total N did not affect the abundance of *nosZ* genes (Fig. 6D and F). This result was in agreement with the results of Wallenstein et al. (2006) and Chroakova et al. (2009), and supports their statement that *nosZ* gene seems to be more cosmopolitan in various soils and less affected by environmental factors than the other genes.

## 5. Conclusions

Our study showed that soil moisture is the most important factor controlling the $N_2O$ emission potential in the meadow-steppe grassland. The abundances of *amoA* and *narG* genes are good predictive variables for the potential biogeochemical rates. The effects of grazing are masked or adjusted by soil moisture conditions. The grassland soils protected from animal grazing or that under a moderate grazing for five years did not show a significant difference in potential $N_2O$ emissions. Our results suggest that grazing induced grassland degradation is not necessarily associated with a reduction in $N_2O$ emissions as reported in other extensively managed grasslands in a more arid environment.

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