Contrasting response of two grassland soils to N addition and moisture levels: N₂O emission and functional gene abundance

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Abstract

Purpose Nitrification and denitrification processes dominate nitrous oxide (N₂O) emission in grassland ecosystems, but their relative contribution as well as the abiotic factors are still not well understood.

Materials and methods Two grassland soils from Duolun in Inner Mongolia, China, and Canterbury in New Zealand were used to quantitatively compare N₂O production and the abundance of bacterial and archaeal *amoA*, denitrifying *nirK* and *nirS* genes in response to N additions (0 and 100 µg NH₄⁺-N g⁻¹ dry soil) and two soil moisture levels (40 and 80 % water holding capacity) using microcosms.

Results and discussion Soil moisture rather than N availability significantly increased the nitrification rate in the Duolun

soil but not in the Canterbury soil. Moreover, N addition promoted denitrification enzyme activities in the Canterbury soil but not in the Duolun soil. The abundance of bacterial and archaeal *amoA* genes significantly increased as soil moisture increased in the Duolun soil, whereas in the Canterbury soil, only the abundance of bacterial *amoA* gene increased. The increase in N₂O flux induced by N addition was significantly greater in the Duolun soil than in the Canterbury soil, suggesting that nitrification may have a dominant role in N₂O emission for the Duolun soil, while denitrification for the Canterbury soil.

Conclusions Microbial processes controlling N₂O emission differed in grassland soils, thus providing important baseline data in terms of global change.

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1 Introduction

The increasing demand for food and energy has profoundly changed the biogeochemical cycle of nitrogen (N). Atmospheric nitrous oxide (N₂O) as a potent greenhouse gas has increased substantially from 270 to 319 parts per billion by volume (ppbv) since the Industrial Revolution (IPCC 2007). Emissions from fertilized fields (4.3–5.8 Tg N₂O–N year⁻¹) and natural unfertilized soils (6–7 Tg N₂O–N year⁻¹) contribute 56–70 % of all global N₂O emissions (Syakila and Kroeze 2011). N₂O was traditionally recognized as one of the by-products of the denitrification process, a stepwise reduction of NO₃⁻ through NO₂⁻, NO, N₂O to N₂ under anaerobic conditions. During the last decade, increasing evidence has shown that both nitrification and denitrification are important processes for N₂O emissions in soil (Wrage et al. 2001).

The microbial metabolic pathways involved in N_2O emission are directly influenced by the amount of ammonium and nitrate in soil as well as other factors. For example, multiple linear regression analysis indicated that ammonium content significantly affected N_2O emissions in New Zealand grazed grassland soils (Carran et al. 1995). Similarly, higher N_2O emissions (above $2 \text{ kg N}_2\text{O-N ha}^{-1} \text{ year}^{-1}$) were found at all sites with N fertilizer application over 150 kg N ha^{-1} across European grassland soils (Flechar et al. 2007). On the other hand, it has been well established that soil moisture is the key driver determining soil N_2O emissions as it regulates oxygen availability to soil micro-organism. Nitrification can contribute a major proportion of N_2O emissions under low O_2 availability using pure ammonia-oxidizing bacteria (Goreau et al. 1980) and from soils with high moisture (60 % WFPS) (Abbasi and Adams 2000). Since N_2O emissions from nitrifier denitrification could be independent of O_2 concentration (Wrage et al. 2001), nitrification and denitrification processes are both equally important to soil N_2O emissions (Bateman and Baggs 2005).

N fertilizer application is one of the important management strategies in terrestrial ecosystem, including the grasslands (Peng et al. 2011). Additionally, grasslands make a significant contribution to N_2O emissions (Mosier et al. 1991), mostly attributed to the application of N fertilizers (e.g., calcium ammonium nitrate, ammonium sulfate) (Singh et al. 2010). Our previous studies on typical semi-arid temperate steppe in Inner Mongolia (China) or nitrogen-rich grassland soils in Canterbury (New Zealand) both indicated that high N loading increased the abundance of ammonia-oxidizing bacteria (AOB) but decreased or no effect on that of ammonia-oxidizing archaea (AOA) (Di et al. 2009; Shen et al. 2011). Therefore, comparison of N_2O emission with these two contrasting grassland soils as well as their controlling factors is imperative to determine the effect of N addition and moisture conditions in the context of global climate change. The aim of this study was to compare the effects of N addition and moisture variation on soil N_2O flux and their relationship to microbe-mediated processes (i.e., nitrification and denitrification) via the quantitative analysis of N-related functional genes (*amoA*, *nirK* and *nirS*). We hypothesized that (1) soil moisture level would be the dominant factor affecting N_2O emissions in grassland soils and (2) nitrification rather than denitrification would dominate N_2O emissions in these ecosystems.

2 Materials and methods

2.1 Soil sampling

Soil samples were taken from Canterbury ($43^\circ 38' 11'' \text{ S}$, $17^\circ 12' 18'' \text{ E}$) in New Zealand and Duolun ($42^\circ 2' 30'' \text{ N}$, $116^\circ 17' 24'' \text{ E}$) County in Inner Mongolia, China, respectively. The

sampling site in Canterbury is dominated by perennial ryegrass (*Lolium perenne* L.) pastures with some white clover (*Trifolium repens* L.). Mean annual precipitation is $\sim 660 \text{ mm}$, and mean monthly soil temperature is 3° C in July (winter) and 16° C in January (summer) (Goh and Bruce 2005). Canterbury soil with a medium texture is developed from glacial outwash and classified as Templeton silty loam (USDA: Udic Haplustepts) with 44.2 % sand, 53.7 % silt, and 2.1 % clay (Di et al. 2010). The site had been farmed for a substantial period of time with intensive irrigation and N fertilization. For the Duolun site in Inner Mongolia, it is mainly covered by *Stipa krylovii* steppe including *S. krylovii*, *Artemisia sieversiana*, *Artemisia frigida*, *Agropyron cristatum*, *Allium bidentatum*, and *Salsola collina* (Zhan et al. 2007). The Duolun site is a typical semi-arid steppe with 385.5 mm mean annual precipitation and monthly mean temperature fluctuating from -17.5° C in January to 18.9° C in July. The mean annual potential evaporation is 1748 mm . The soil has a moderately coarse texture with 62.8 % sand, 20.3 % silt, and 16.9 % (sandy loam) and is classified (USDA) as a Typic Haplocalcids. The Duolun soil was collected from fallow land without any irrigation and N fertilization. Both soils were composites of about 5 kg formed from six subsamples taken from an area of 4000 m^2 . These two soils were collected in November of 2011, sealed and kept on ice before transporting to our lab. Soil samples were sieved through a 2-mm mesh and stored at 4° C before establishing the incubation experiment.

2.2 Soil microcosm construction

Soil water-holding capacity (WHC) was measured as described by Klute (2003). Soil WHC was 48.7 and 51.4 % for Duolun and Canterbury soils, respectively. Both soils were pre-incubated at 25° C for 1 week with the moisture adjusted to 40 % WHC with distilled water. Soil microcosms were established in 250-ml serum bottles containing 25 g of pre-incubated soils. The water content was adjusted to 40 and 80 % WHC (hereinafter referred to as LW and HW, respectively) with distilled water using a syringe before N addition. Each soil had two levels of nitrogen fertilizer application (0 and $100 \mu\text{g NH}_4^+-\text{N g}^{-1}$ dry soil and hereinafter referred to as U and N, respectively) with ammonium nitrate (NH_4NO_3). Totally, there are four treatments: LWU, LWN, HWU, and HWN. Sample bottles were partially refreshed by N_2O -free air (N_2 , 79.97 %; O_2 , 20 %; CO_2 , 0.03 %) through gas sampling and during the whole incubation time and incubated at 25° C in the dark. A $100\text{-}\mu\text{l}$ gas volume was taken from each bottle before each sampling. According to previous studies about the short-term effect of ammonium concentration on ammonia-oxidizing bacteria (Okano et al. 2004; Cavagnaro et al. 2008), triplicates for each treatment were destructively

sampled after incubating for 2, 4, 6, 8, 10, 12, and 14 days. Soil samples for measurement of basic properties and DNA extraction were immediately stored at 4 and -80°C , respectively.

2.3 Soil chemical properties, potential nitrification rate, and denitrification enzyme activity

Ammonium and nitrate concentration were determined by a Continuous Flow Analyzer (SAN++, Skalar, Breda, Holland) after extraction of soil samples with 2 M KCl. Soil pH was determined (1:2.5) by a Delta 320 pH-meter (Mettler-Toledo Instruments Co., Shanghai, China). Soil-dissolved organic C (DOC) and N (DON) were extracted by 2 M KCl solution and determined by Liqui TOC analyzer (Elementar, Germany) and persulfate oxidation, respectively (Jones and Willett 2006). Potential nitrification rate (PNR) was measured following the method of Kurola et al. (2005). In brief, 5.0 g (fresh weight) soil sample was incubated in a 50-ml centrifuge tube containing 20 ml of phosphate buffer solution (PBS) (g l^{-1} : NaCl, 8.0; KCl, 0.2; Na_2HPO_4 , 0.2; NaH_2PO_4 , 0.2; pH 7.4) with 1 mM $(\text{NH}_4)_2\text{SO}_4$ and 10 mM KClO_3 . The mixture was incubated at 25°C and shaken at 150 rpm in the dark for 24 h. After extraction with 5 ml of 2 M KCl solution, the nitrite concentration of the supernatant was determined spectrophotometrically at 540 nm with *N*-(1-naphthyl) ethylenediamine dihydrochloride. The process to determine soil denitrification enzyme activity (DEA) is as follows (Barton et al. 2000): 5 g fresh soil was weighed into a glass serum bottle (120 ml) and sealed with a rubber septum and an aluminum crimp cap. Each bottle was flushed with high-purity N_2 gas and amended with a 10-ml solution containing 1 mM glucose, 1 mM potassium nitrate, and 0.39 mM chloramphenicol. Approximately 15 % (V/V) acetylene gas was added to inhibit N_2O reduction. Thirty milliliters of headspace was sampled and stored in a 200-ml gas sampling bags before incubation. Bottles were shaken at room temperature for 30 min and then incubated at 25°C for 1 h. Another 30 ml of headspace was collected and stored for N_2O analysis.

2.4 N_2O flux

Gas sampling was taken every 48 h during the incubation period. Exactly 100 ml pure air (N_2 , 79.97 %; O_2 , 20 %; CO_2 , 0.03 %) was mixed with the headspace of the bottles and then 100 ml mixed gas was collected and stored in a 200-ml gas sampling bags for N_2O analysis. The N_2O flux was calculated using the N_2O concentrations measured every 2 days, i.e., at 2, 4, 6, 8,

10, and 14 days. N_2O concentration was analyzed with a gas chromatograph (Agilent 7890B) equipped with an electron capture detector (ECD).

2.5 DNA extraction and quantitative analysis of N-related functional genes

Soil DNA was extracted from 0.5 g (wet weight) samples using the FastDNA Spin Kit (for soil; Bio 101, Carlsbad, CA) in accordance with the manufacturer's instructions. The concentration of extracted DNA was measured by taking A260 and A280 on a NanoDrop-2000 UV-Vis Spectrophotometer (Thermo Scientific). Quality checking of DNA was examined by 1 % agarose gel electrophoresis.

Soil samples after incubating for 2, 6, 10, and 14 days were chosen for the quantitative PCR (Q-PCR) analysis. Bacterial and archaeal ammonia monooxygenase (*amoA*) and nitrite reductase (*nirK* and *nirS*) genes were determined in triplicate on an iCycler IQ 5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). Primer pairs *amoA*1F (5'-GGG GTT TCT ACT GGT GGT-3')/*amoA*2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') (Rotthauwe et al. 1997) and *CrenamoA*23F (5'-ATG GTC TGG CTW AGA CG-3')/*CrenamoA*616R (5'-GCC ATC CAT CTG TAT GTC CA-3') (Tournai et al. 2008) were used to quantify bacterial and archaeal *amoA* genes, respectively, as described previously (Long et al. 2012). The quantitative analysis of *nirK* gene (473 bp) was performed with the primer set *FlaCu* (5'-ATC ATG GTS CTG CCG CG-3')/*R3Cu* (5'-GCC TCG ATC AGR TTG TGG TT-3') (Garbeva et al. 2007). The thermal cycling conditions were 95°C for 5 min, 40 cycles of 15 s at 95°C , 45 s at 57°C , and 30 s at 72°C . The abundance of *nirS* gene was detected using the primers *nirS*1F (5'-CCT AYT GGC CGC CRC ART-3') and *nirS*6R (5'-CGT TGA ACT TRC CGG T-3') (Braker et al. 1998) producing an 890-bp product. Amplification conditions were as follows: 95°C for 10 min, 40 cycles of 10 s at 95°C , 30 s at 60°C , and 45 s at 72°C . To eliminate the accumulation of primer dimers and improve the specific products, the fluorescence signal was detected at 81, 83, 88, and 90°C for bacterial *amoA*, archaeal *amoA*, *nirK*, and *nirS* genes, respectively. The Q-PCR reaction mix contained 10 μl SYBR® Premix Ex Taq™ (Perfect Real Time; Takara Bio), 200 μM of each primer, 2 μl of 10-fold dilution DNA template (10–20 ng), and ultraclean water to 20 μl total volume. The inhibitory effects of the coextracted substances in soil DNA samples were also tested by determining a dilution series of 10-, 20-, and 50-fold of the extracted DNA in the presence and absence of bovine serum albumin

(BSA). Plasmid DNA for the respective functional gene was extracted from the clones of environmental DNA and serially diluted to generate a standard curve as described previously (Long et al. 2012). The corresponding Q-PCR efficiency was 90–105 % and r^2 was 0.94–1.01.

2.6 Statistical analysis

In order to minimize the soil heterogeneous influence on this experiment, we subdivided the composite samples to three sub-sample as triplicates, which is traditionally treated as pseudo-replication, in which experimental units are not naturally independent (Hurlbert 1984). However, mixed-effect models can be used to soften this problem (Crawley 2007).

We used R (R Core Team) and lme4 (Bates et al. 2012) to perform a linear mixed-effect analysis of the effects of soil moisture and N fertilization, which were defined as fixed factors, whereas the sample and time were defined as random factors. Visual inspection of residual plots and Shapiro-Wilk W test was performed to test the homoscedasticity or normality of the variables. Correlations between soil properties and the abundance of key genes (*amoA*, *nirK*, *nirS*) involved in nitrification and denitrification processes were analyzed using the Spearman rank correlation test. Duncan's multiple range test was used to determine the difference of N-related functional gene abundance between the four treatments. Statistically significant differences were tested at p values <0.05.

3 Results

3.1 Soil chemical properties

Ammonium and nitrate levels showed a similar trend in both grassland soils, i.e., the highest concentrations were found in the N addition treatments (i.e., LWN and HWN), while the lowest in the control treatments (i.e., LWU and HWU) (Table S1, Electronic supplementary material). The NH_4^+ -N concentration decreased significantly in the low water treatments for the Canterbury soil and in the N addition treatments for the Duolun soil (Table S1, Electronic supplementary material). In comparison with the initial condition of incubation (day 2), the NO_3^- -N concentration increased significantly in all the treatments of both Duolun and Canterbury soils after 14-day incubation, except the LWU treatments. Meanwhile, the DOC content decreased dramatically in all the treatments in the Canterbury soil along the incubation period, while kept constant in the Duolun soil (Table S1, Electronic supplementary material). Along

the incubation period, the DON content increased in the treatments with N addition in both soils, while kept stable for no N addition treatments. Soil pH decreased in all the treatments for both soils along the incubation period with more obvious trends in N addition treatments.

3.2 Potential nitrification rates, denitrification enzyme activity, and N_2O flux

The PNR responded differently to soil moisture levels for the two grassland soils. For example, in the Duolun soil, PNR increased significantly with the increase of soil moisture (Fig. 1). However, the increase of soil moisture showed a negative effect in the Canterbury soil. The DEA in the HWN treatment was higher than that of other treatments in the Canterbury soil, whereas DEA content in LWU and HWU treatments began to increase after incubating for 1 week (Fig. 2). However, the DEA in the Duolun soil did not significantly change during the whole incubation period with the exception of the HWU treatment, which had a higher level at the later period.

Soil N_2O flux in the N addition treatments was significantly higher than those of the no N addition treatment, which decreased significantly during the incubation period in both soils (Fig. 3). Interestingly, the N_2O fluxes of the Duolun soil in the N addition treatments were higher than that of Canterbury soil. However, the N_2O fluxes tended to be higher in the LWN treatment than HWN treatment in both grassland soils. There was a significant interactive effect between soil moisture and N addition on N_2O flux in Canterbury soil but only soil moisture significant effect was observed in Duolun soil (Table 1). The mixed-effect regression analysis showed that the dominant contributors to the N_2O flux were soil DEA and PNR for the Canterbury soil, while ammonium, nitrate, and DEA for the Duolun soil (Table 2).

3.3 The abundance of bacterial *amoA*, archaeal *amoA*, *nirK*, and *nirS* genes

Soil moisture and N addition significantly affected the abundance of nitrifiers but not denitrifiers in both Canterbury (Fig. S1, Electronic supplementary material) and Duolun soils (Fig. S2, Electronic supplementary material). For the Canterbury soil, N addition tended to stimulate the growth of ammonia oxidizers in 40 % WHC condition and showed a negative effect in 80 % WHC condition. In contrast, the positive effect of N addition on AOB increased significantly with the increasing of soil moisture in Duolun soil. However, little variation in *nirK* and *nirS* abundances was found among

Fig. 1 Potential nitrification rates of the Canterbury and Duolun soils under different levels of N addition (0 (L) and $100 \mu\text{g NH}_4^+-\text{N g}^{-1}$ dry soil weight (N)) and soil moisture (40% (LW) and 80% WHC (HW)) during 14 days incubation

the treatments in both soils after 14 days incubation. Two-way ANOVA analysis showed soil moisture significantly affected the abundance of the bacterial *amoA* gene in both soils and archaeal *amoA* gene in Duolun soil (Table 1). Meanwhile, there was a significant effect of soil moisture on *nirK* gene for Duolun soil and *nirS* gene for Canterbury soil. On the other hand, nitrogen addition profoundly affected the abundance of *nirK* and *nirS* genes in the Canterbury soil, but not significant in the Duolun soil based on the two-way ANOVA analysis. Evidence from Spearman rank correlation analysis also revealed different relationships between functional gene abundance and soil chemical properties in these two grassland soils (Table 3). For example, there was a significant negative correlation between ammonium concentration and the abundance of archaeal *amoA*, *nirK*, and *nirS* genes in the Canterbury soil but not in the Duolun soil. Moreover, soil DOC was found to be significantly positively correlated with the abundance of archaeal and bacterial *amoA* and *nirK* genes in the

Fig. 2 Denitrification enzyme activities of the Canterbury and Duolun soils under different levels of N addition (0 (L) and $100 \mu\text{g NH}_4^+-\text{N g}^{-1}$ dry soil weight (N)) and soil moisture (40% (LW) and 80% WHC (HW)) during 14 days incubation

Duolun soil but only bacterial *amoA* gene in the Canterbury soil.

4 Discussion

4.1 Nitrifiers responded differently to increasing soil moisture and N addition

Microbes in Canterbury and Duolun soils responded distinctively to increasing soil moisture and N fertilizer addition, indicating that N transformation processes varied with different grassland soil types. PNR decreased significantly under the wetter condition (80% WHC) in the Canterbury soil, while increased in the Duolun soil. A previous study also found that nitrification increased with increasing soil moisture (from -1500 to -33 kPa) but ceased under water-saturated conditions (0 kPa) (Malhi and McGill 1982). The increase in soil water content may improve the substrate available for microbes, especially in a semi-arid soil with a moderately

Fig. 3 Nitrous oxide flux of the Canterbury and Duolun soils under different levels of N addition (0 (U) and 100 $\mu\text{g NH}_4^+\text{-N g}^{-1}$ dry soil weight (N)) and soil water contents (40 % (LW) and 80 % WHC (HW)) during 14 days incubation

coarse texture such as the Inner Mongolia steppe soil (Austin et al. 2004). Moreover, net nitrification indicated a positive effect of N addition on the nitrification process after 14-day incubation. However, net nitrification was found to increase with low $\text{NH}_4^+\text{-N}$ input but be depressed with high $\text{NH}_4^+\text{-N}$ (300 $\mu\text{g g}^{-1}$) treatment (Malhi and McGill 1982).

Nitrogen application had been found to stimulate the growth of AOB but not AOA in various soil microcosm studies (Di et al. 2009, 2010; Verhamme et al. 2011), due to their specific preference for ammonia. In comparison with no N addition treatment, the abundance of AOB increased significantly with N addition in the Duolun soil under 80 % WHC condition but was observed in the Canterbury soil under 40 % WHC condition. Stimulation of N input on AOB growth had been reported in a short-term incubation experiment (Okano et al. 2004), and a long-term N addition experiment in Inner Mongolia steppe grassland (Shen et al. 2011). In contrast, the

Table 1 Linear mixed-effect models testing effect of soil moisture and N fertilization on the abundance of bacterial *amoA* (AOB), archaeal *amoA* (AOA), *nirK*, *nirS* genes, and N_2O flux (N_2O) ($n = 48$)

fixed effects	df	p values				
		N ₂ O	AOB	AOA	nirK	nirS
Canterbury						
Soil moisture (M)	1	<0.001	0.050	0.077	0.933	0.003
N fertilization (N)	1	<0.001	0.924	0.158	0.049	0.030
M × N	1	<0.001	0.908	0.006	0.559	<0.001
Shapiro-Wilk normality test of residuals						
p value		0.08	0.08	0.01	0.38	0.59
Duolun						
Soil moisture (M)	1	<0.001	0.046	0.046	0.026	0.400
N fertilization (N)	1	0.909	0.519	0.373	0.634	0.329
M × N	1	0.923	0.021	0.787	0.423	0.125
Shapiro-Wilk normality test of residuals						
p value		0.11	0.56	0.34	<0.01	0.02

abundance of AOA in this study tended to decrease under high N input, which was consistent with the study in a greenhouse-based high N input vegetable soil (Zhong et al. 2016). The differential response of AOA to N addition was mainly due to its specific preferences for low ammonia as demonstrated by studies via pure cultures and microcosm experiments (Martens-Habben et al. 2009; Shen et al. 2014).

Our results showed that soil moisture significantly promoted the growth of AOB in the Duolun and Canterbury soils, indicating that soil moisture was another major driver influencing AOB, especially for semi-arid ecosystems (Austin et al. 2004). The Canterbury pasture soil was regularly

Table 2 Mixed-effect regression models analysis for N_2O emission in the Canterbury and Duolun soils and sampling time as the random variable ($n = 48$)

Fixed effects	Canterbury		Duolun	
	Standard error	p value	Standard error	p value
DEA	<0.01	0.007	0.05	0.007
PNR	0.26	<0.001		
$\text{NH}_4^+\text{-N}$			<0.01	<0.001
$\text{NO}_3^-\text{-N}$			<0.01	<0.001
Information criteria				
AIC	55.77		102.09	
BIC	65.12		113.31	
Shapiro-Wilk normality test of residuals				
p value	0.07		0.42	

AIC Akaike information criterion, BIC Bayesian information criterion

Table 3 Spearman rank correlation between soil physiochemical properties and functional gene abundance ($n = 48$)

Genes	NH_4^+-N	NO_3^--N	DOC	DON	pH
Canterbury					
Bacterial <i>amoA</i>	0.15	0.17	0.53***	0.09	0.48***
Archaeal <i>amoA</i>	-0.36**	-0.13	-0.14	-0.22	0.01
<i>nirK</i>	-0.34**	-0.37**	0.21	-0.32*	0.61***
<i>nirS</i>	-0.53***	-0.49***	-0.09**	-0.48***	0.26
Duolun					
Bacterial <i>amoA</i>	0.28	0.53***	0.41***	0.28	-0.16
Archaeal <i>amoA</i>	-0.10	0.03	0.48***	0.08	0.29*
<i>nirK</i>	-0.06	0.07	0.38***	0.01	0.09
<i>nirS</i>	-0.14	-0.37**	-0.12	-0.24	0.28

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ —stastically significant values

irrigated with water and maintained a moderate soil moisture condition (40 % WHC, 20.5 % water content), whereas the natural Duolun soil had a relative lower water content (14 % WHC, 6.5 % water content). The different response of AOB to the N addition between the Canterbury soil and the Duolun soil under the same moisture condition might be attributed to the limitation of the ammonium availability or nutrient diffusion, respectively. The diffusional limitations of ammonia and cell dehydration had been demonstrated to inhibit the activity of AOB in soil with low water content (Stark and Firestone 1995). Increasing soil water content generally improved the availability of substrate for microbes and addition of water to a dry soil initiates a flush of C and net N mineralization (the “Birch effect”) (Fierer and Schimel 2003). Similarly, Chen et al. (2013) also found that increased precipitation had a positive effect of on the abundance of AOB but not AOA in our sampling site. The different response of AOA to soil moisture in this study from that of Chen et al. (2013) could be ascribed to the fact that the stimulation of precipitation might be softened due to water evaporation and infiltration in the field.

4.2 Denitrifiers responded differently to increasing soil moisture and N addition

For the DEA analysis, N fertilizer input showed a significant effect on DEA based on the statistical analysis but without a uniform pattern for both soils. A Michaelis-Menten kinetics model between denitrification activity and nitrate concentration had been discussed as a type-I or type-II response, where denitrification were determined by nitrate concentration or available organic carbon (Strong and Fillery 2002). The effect of N fertilizer on the abundance of *nirK* and *nirS* genes in the Duolun soils was not significant, which was consistent with

the study of Szukics et al. (2009), who found no significant change in the abundance of the *nirK* gene in Rothwald and Schottenwald soils with the application of 0.1 mg $(\text{NH}_4)_2\text{SO}_4\text{-N g}^{-1}$ or 0.1 mg $\text{KNO}_3\text{-N g}^{-1}$ dsw. Szukics et al. (2009) further suggested that organic carbon dominated the growth of denitrifiers and denitrification activity but was not necessarily related to the denitrifiers population size. Similarly, Wallenstein (2004) found that in a long-term N fertilization experiment, the abundance of *nirK* and *nirS* genes was lower in the fertilized plots compared with control plots. In contrast, long-term fertilized paddy soil with balanced mineral fertilizer and urea only showed an increase of *nirK* and *nirS* genes abundances (Chen et al. 2010). The grazed pasture ecosystem in the Canterbury soil is high in organic matter (3–10 %) (Haynes and Williams 1993) and rich in topsoil N (Di et al. 2009), which was significantly different from the soil properties of Duolun soil. The abundance of the *nirK* and *nirS* genes were negatively correlated with ammonium and nitrate concentrations for the Canterbury soil, however, the *nirS* gene abundance was negatively correlated with nitrate in Duolun soil. Although nitrate availability is known to exert a strong control on denitrification rates, experimental data of fluorescent DNA probe hybridization showed that there was no significant correlation between denitrifier abundance and nitrate concentrations in an acidic forest soil depth profile (Mergel et al. 2001). However, we found a positive correlation between *nirK* abundance with DOC in the Duolun soil. Wallenstein et al. (2006) proposed that factors including pH, moisture and O_2 availability other than substrate supply primarily controlled denitrifier community abundance.

In comparison with 40 % WHC condition, increasing soil moisture tended to increase the abundance of the *nirK* gene in Duolun soil. Similarly, *nirK* gene abundance increased with soil moisture gradient and was limited by the substrate (NO_3^--N) availability in a microcosm experiment at 5 and 15 °C (Szukics et al. 2010). In contrast, increasing soil moisture in combination with N addition would significantly decrease the abundance of *nirS* gene in Canterbury soil. However, the *nirS*-type denitrifiers were thought to adapt to environments with high water contents in a waterlogged boreal forest soil (Petersen et al. 2012) and were found to be positively related to soil moisture in a grassland pasture (Philippot et al. 2009). Divergent results between this study and previous researches mainly attributed to different community of *nirK* and *nirS* denitrifiers from each environment.

4.3 N_2O fluxes responded differently to increasing soil moisture and N addition

Soil moisture and N addition would significantly change the substrate availability and O_2 partial pressure, which regulating the N_2O production in soil. A study in wheat and barley field soils indicated that nitrification was the dominant contributor

to N_2O emission at soil moisture contents lower than 90 and 80 % WHC, respectively (Bollmann and Conrad 1998). Szukics et al. (2009) also found nitrification was the main source of N_2O in the Rothwald forest soil at 70 % WFPS with NH_4^+-N application. More recently, Zhu et al. (2013) found the NH_3 oxidation pathway dominated N_2O production even at 0.5 % oxygen availability. However, it has been demonstrated that nitrification and denitrification may occur simultaneously in soils (Davidson et al. 2000). In our study, a positive relationship between N_2O flux and soil ammonium concentration in both tested soils indicated nitrification may dominate the N_2O emission in soil. Similarly, Mummey et al. (1994) found nitrification dominated the sources of N_2O in a semi-arid shrub steppe in North America along a gradient of soil moisture, but was surpassed by denitrification under saturated soil conditions. A significant effect of both DEA and PNR on the soil N_2O emission in the Canterbury soil indicated denitrification also made a contribution to the N_2O production. In comparison, N_2O flux tended to be affected by the availability of organic carbon in the Duolun soil. The accumulation of nitrate in soil microsites may decrease soil pH and inhibit nitrification and denitrification (Mulaney et al. 1997; De Boer and Kowalchuk 2001). Consequently, we suggested that N_2O emissions can be driven by the nitrification process in these two typical grassland soils, but the N cycling pattern may be distinctive for each soil.

5 Conclusions

In summary, this study revealed a distinct response of two grassland soils to the treatments with N addition in combination with increased soil moisture during a short-term incubation. Soil moisture rather than the availability of ammonium increased the nitrification rate in Duolun soil. In contrast, the increasing of soil moisture stimulated denitrification enzyme activities in Canterbury soil. The increase in the N_2O flux induced by N addition was significantly greater in the Duolun soil than in the Canterbury soil. Differential responses of N_2O emission to the treatments in these two grassland soils indicated that nitrification may have a dominant role in N_2O emissions for the Duolun soil, while denitrification had the dominant role in the Canterbury soil, suggesting that both processes need to be considered when assessing N_2O emissions from grassland soils in terms of global change. Stable isotopic probing techniques could provide more direct evidence to identify the dominant contributor to the N_2O production in grassland soils in the future.

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