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ORIGINAL PAPER

Microbial pathways for nitrous oxide emissions from sheep urine and dung in a typical steppe grassland

Hong Pan[1] · Shanshan Ying[1] · Haiyang Liu[1] · Lingzao Zeng[1,2] · Qichun Zhang[1,2] · Yimeng Liu[3] · Jianming Xu[1,2] · Yong Li[1,2] · Hongjie Di[1,2]

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Abstract

The aim of this study was to determine the responses of nitrifiers and denitrifiers to understand microbial pathways of nitrous oxide (N_2O) emissions in grassland soils that received inputs of sheep excreta. Sheep dung and synthetic sheep urine were applied at three different rates, simulating a single, double, or triple overlapping of urine or dung depositions in the field. Quantitative PCR and high-throughput sequencing were combined with process-based modeling to understand effects of sheep excreta on microbial populations and on pathways for N_2O production. Results showed that emissions of N_2O from urine were significantly higher than from dung, ranging from 0.12 to 0.78 kg N_2O-N ha^{-1} during the 3 months. The N_2O emissions were significantly related to the bacterial *amoA* ($r = 0.373$, $P < 0.001$) and *nirK* ($r = 0.614$, $P < 0.001$) gene abundances. It was autotrophic nitrification that dominated N_2O production in the low urine-N rate soils, whereas it was denitrification (including nitrifier denitrification and heterotrophic denitrification) that dominated N_2O production in the high urine-N rate soils. Nitrifier denitrification was responsible for most of the N_2O emissions in the dung-treated soils. This study suggests that nitrifier denitrification is indeed an important pathway for N_2O emissions in these low fertility and dry grazed grassland ecosystems.

Keywords Microbial pathway · Nitrous oxide · Nitrifier · Denitrifier · Excreta

Introduction

Grassland, as the largest terrestrial ecosystem in the world, accounts for more than 40% of China's total land area (Nan 2005). To meet the food demands of a growing population in China, livestock stock densities in these grasslands are increasing. Heavy grazing by higher stocking rates can result in grassland degradation. At the same time, higher stocking rates also create excrement "hot spots," which are well-known sources of nitrous oxide (N_2O) emissions (Bol et al. 2004; Saggar et al. 2004; Cardenas et al. 2007).

Nitrous oxide is a potent greenhouse gas, contributing up to 6% of global warming and is also an ozone depletion gas (Loick et al. 2017). Its concentration has steadily increased at a rate of 0.73 ± 0.03 ppb $year^{-1}$ over the last three decades (IPCC 2014). N_2O is traditionally thought to be mainly produced by autotrophic nitrification and heterotrophic denitrification in the soil (Zhong et al. 2014). The conversion of ammonia (NH_3) to nitrite (NO_2^-), which is the first and rate-limiting step of nitrification, is catalyzed by the ammonia monooxygenase (AMO) enzyme, which is encoded by the *amoA* genes of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Venter et al. 2004; Francis et al. 2005; Könneke et al. 2005; Wuchter et al. 2006; Prosser and Nicol 2008). Denitrification consists of the sequential reduction of NO_3^- to N_2 via the metalloenzymes nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, with N_2O as an intermediate product (Zumft 1997; Philippot and Hallin 2005). Another important

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✉ Yong Li
liyongcn@zju.edu.cn; yongl@zju.edu.cn

[1] Institute of Soil and Water Resources and Environmental Science, College of Environmental and Resource Sciences, Zhejiang University, Yuhangtang Road No. 866, Hangzhou 310058, China

[2] Zhejiang Provincial Key Laboratory of Agricultural Resources and Environment, Hangzhou 310058, China

[3] School of Economics and Resource Management, Beijing Normal University, Beijing 100875, China

biotransformation process for N_2O production is nitrifier denitrification, and this process may play an important part in the emissions of N_2O under some conditions, e.g., soil moisture conditions that are sub-optimal for denitrification (Webster and Hopkins 1996; Kool et al. 2011). Nitrifier denitrification is the oxidation of ammonia (NH_3) to NO_2^- followed by the reduction of NO_2^- to N_2O via NO (Wrage et al. 2001; Stein 2011). As sole reductases in the nitrifier denitrification, a diverse group of *nirK* and *norB* genes have been detected in numerous AOB species, which are thus far the only group of microorganisms performing nitrifier denitrification (Stein 2011).

Urea-N is the main form of N in sheep urine returned to soil (Di and Cameron 2002; Shand et al. 2002), and excessive N fertilization had the potential to promote N_2O emissions via inhibiting the activity of *nosZ* genes (Qin et al. 2017). AOA dominated nitrification in lightly grazed soils while AOB dominated nitrification in heavily grazed systems (Pan et al. 2018). AOB contributed more than AOA to N_2O emissions from NH_4^+-N applied arable soils (Wang et al. 2016). Urine application dramatically increased the abundance of AOB *amoA*, *nirK*, and *nosZ* genes, stimulated N_2O emissions, and decreased the abundance of AOA in grassland systems (Di et al. 2010, 2014). Fresh dung treatments significantly increased N_2O emissions (Ma et al. 2006; Lin et al. 2009) and enhanced soil N retention (Cheng et al. 2016). Wrage et al. (2004) found that application of artificial sheep urine altered the microbial pathways for N_2O emissions, and urine significantly promoted N_2O production by nitrifier denitrification in an incubation experiment. Additionally, sheep urine and dung tended to overlap in the areas where livestock frequently congregate (such as around drinking water troughs, animal tracks) or in pastures with high stocking rates. The effects of overlapping urine or dung patches on N_2O emissions with specific N-cycling pathways in the field are poorly understood.

Therefore, a field experiment that lasted from summer to autumn was conducted in an Inner Mongolia typical grassland. The rainfall in this area is low and falls mainly during July–September which coincides with the plant growing season, and grazing management usually happens during this period. The aim of this study was to improve our understanding of the contribution of the three processes (nitrifier denitrification, autotrophic nitrification and heterotrophic denitrification) to N_2O emissions by a combination of molecular biology techniques and processed-based model analysis. We hypothesized that (1) nitrifier denitrification was an important pathway for the production of N_2O in grazed grassland soils; (2) N_2O emissions from urine and dung patches would be much higher than those without sheep excreta; and (3) AOA would play an important role in these low fertility and dry grassland environments.

Materials and methods

Site description

The field experiment was conducted in Xilingol region Chaokewula Sumu (44° 15″ 24.43′–44° 15″ 40.66′ N, 116° 32″ 8.16′–116° 32″ 28.32′ E) in Inner Mongolia, China, from August 5 to November 4, 2014. The region is located at an attitude of 1111–1121 m above sea level and has a typical continental monsoon climate. This region is very cold in winter and cool in summer. The mean annual temperature is − 0.1 °C, with the minimum and maximum monthly mean temperatures ranging from − 22 °C in January to 18.3 °C in July. The average annual precipitation is 350–450 mm, distributed unevenly among seasons, falling mainly during July–September. The annual potential evapotranspiration ranges from 1600 to 1800 mm. The experiment was set in a fenced area of the typical steppe, which is dominated by *Leymus chinensis* and some *Stipa krylovii*, *Stipa grandis*, and *Cleistogenes squarrosa*. The soil type is dark chestnut (calcic Chernozem according to ISSS Working Group RB, 1998) (Bai et al. 2010). The initial properties of the surface soil (Chestnut soil) (0–10 cm) were as follows: soil pH (soil:H_2O ratio 1:2.5) 7.57; total C (TC) and N (TN) contents 14.699 and 0.950 g kg^{-1}, respectively; soil organic carbon (SOC) 15.84 g kg^{-1}; available phosphorus (Olsen P) 1.158 mg kg^{-1}; available potassium (AK) 156.75 mg kg^{-1}; soil cation exchange capacity (CEC) 15.84 cmol kg^{-1}; and soil bulk density 1.32 g cm^{-3}.

Experiment design and treatment application

Fresh sheep dung was collected every morning (about 4–5 a.m.) for 5 days and kept in a − 20 °C freezer until used. Synthetic sheep urine was prepared according to Shand et al. (2002) before application. The average composition of the fresh dung was 438.037 g total C kg^{-1} and 15.540 g total N kg^{-1}.

Forty static chambers (50 cm diameter) divided into two rows of rings were installed into the soil to a depth of 10 cm. Another 40 plots of the same size next to the gas rings were used for soil sampling. Therefore, 80 50-cm-diameter plots were set up as described above. A 1-m-wide buffer strip was established between every two plots to avoid interactions and to allow for sampling the plots.

Ten treatments, each with four replicates, were allocated to the plots in a completely randomized design. According to Ma et al. (2006), one adult sheep usually urinates about 70 mL and excretes 60 g at a time and forms a urine patch about 320 cm^2 and a dung patch about 400cm^2 at a time, respectively, based on the field survey. The treatments included the following: control (CK, nothing was applied); U1, 0.4375 L synthetic sheep urine; W1, 0.4375 L water, to check the effect of water

without N, consistent with the U1 treatment; U2, 0.875 L synthetic sheep urine; W2, 0.875 L water, consistent with the U2 treatment; U3, 1.3125 L synthetic sheep urine; W3, 1.3125 L water, consistent with the U3 treatment; D1, 300 g fresh dung; D2, 600 g fresh dung; and D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively. The urine was applied with a watering can at 80 cm above the soil surface to resemble a real urination of adult sheep. The fresh dung was applied by evenly spreading it over the surface. The higher rates of urine and dung were applied to simulate situations where urine or dung patches were overlapping on top of each other two or three times.

Gas samples and soil samples were collected 12 times during the 91-day field study (0, 1, 3, 7, 14, 21, 28, 42, 56, 61, 70, 91 days, respectively). Gas samples (40 mL) were collected 0 and 40 min after chamber closure by a 20-mL syringe and injected into pre-evacuated 20-mL glass bottles and were collected during 10:00 a.m.–12:00 noon. A gas chromatograph (Shimadzu GC-2010 Plus, Japan) equipped with an electron capture detector (ECD) was used to analyze the concentrations of N_2O. The hourly N_2O emissions were calculated using the equation detailed in the Supplementary material.

Soil samples were collected from the upper 10 cm layer from three random locations within each corresponding plot. The three samples from each plot were bulked into a single composite sample, packed with ice packs, and transported to the laboratory.

Extraction of total DNA from soils and quantitative PCR of functional genes

DNA was extracted from 0.5 g (fresh weight) of soil with a FastDNA Spin Kit for Soil (MP Biomedicals, LLC., Solon, OH, USA), in accordance with the manufacturer's protocol. The DNA size and integrity were checked by electrophoresis on a 0.7% agarose gel; the quantity and purity were estimated using a Nanodrop®ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted DNA was stored at − 20 °C until analysis.

Quantitative PCR assays targeting *amoA* gene of AOA and AOB, bacterial *nirS*, *nirK* and *nosZ* genes were carried out in triplicate on a LightCycler 480 (Roche Applied Science) machine. The standard curves were developed as described previously by Di et al. (2009). Each gene fragment was PCR-amplified with primers and PCR conditions detailed in Table S1. Triplicate PCR amplicons were subsequently pooled and purified with a PCR cleanup kit (TransGen Biotech, Beijing, China) before being cloned into the pGEM-T Easy Vector (TransGen Biotech, Beijing, China). The resulting ligation mix was transformed in *Escherichia coli JM109* competent cells (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The correct inserts were checked by re-amplification of each target gene and positive ones were sequenced with an ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), after cycle sequencing reactions using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The concentrations of plasmid DNA were measured using a Nanodrop®ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The known copy number plasmid DNA was 10-fold serial diluted to generate the standard curve.

Each 20 μL reaction mixture contained 10 μL SYBR Premix Ex Taq (TaKaRa, Dalian, China), 400–750 nM of each primer, 1 μL of 10-fold diluted DNA template (1–10 ng), and 8.4–8.68 μL Milli-Q water. Melting curve analysis was performed at the end of each quantitative PCR run to confirm PCR product specificity by measuring fluorescence continuously with the temperature increasing from 50 to 99 °C. High efficiencies of 87.7–101.1% were obtained for the functional genes amplification, with the R^2 values ranging between 0.965 and 0.998.

Cloning, MiSeq sequencing, and phylogenetic analysis

The cloning procedure was similar to that described above for generating standard curves for qPCR analyses. The difference was that positive inserts of each target gene (each containing 25 clones) were sequenced. The sequences were clustered into operational taxonomic unit (OTU) at 97% cutoff using the mothur software (Schloss et al. 2009).

High-throughput sequencing of 16S rRNA genes encompassing the V4 region was conducted with a universal 515F-907R primer of the soil DNA using the Illumina® MiSeq sequencer (Illumina, San Diego, CA, USA). In this study, only sequences > 200 bp in length, with an average quality score > 25, without ambiguous base calls, match the primer and present with at least an 80% match to a previously determined 16S rRNA gene sequences were included in the subsequent analyses. The trimmed and unique sequences were clustered into operational taxonomic units (OTUs) based on 97% pairwise identity.

A representative sequence was then used from each OTU for phylogenetic analysis. The phylogenetic analyses of archaeal and bacterial 16S rRNA as well as *nirK* gene were then conducted by Molecular Evolutionary Genetic Analysis software (MEGA6.06) with 1000-fold bootstrap support. The DNA sequences used for phylogenetic tree construction were deposited in the DNA Data Bank of Japan (DDBJ) under the accession number LC217919-LC217983 (*nirK*). All the raw sequence data of 16S rRNA has been deposited to NCBI's Sequence Read Archive (SRA) under accession number SRP101303.

Process-based model description

In order to evaluate the contributions of the three predominant biotransformation processes (nitrifier denitrification, autotrophic nitrification and heterotrophic denitrification) to N_2O production, N_2O in soil (N_2O soil) was divided into two pools (N_2O soil-1 and N_2O soil-2) due to independent microbe activity (Baggs 2011). The other N pools were exchangeable NH_4^+-N, NO_3^--N, N_2 and N_2O air.

As shown in Fig. S1, the oxidation of NH_4^+ to N_2O soil-2 represented the N_2O emissions from autotrophic nitrification and nitrifier denitrification, which are important biotransformation processes for N_2O production in soils (Kool et al. 2010). Heterotrophic denitrification comprised the reduction from NO_3^- to N_2O soil-1 by *nirK* and *nirS*, and reduction from N_2O soil-1 to N_2 by *nosZ*. Ammonia volatilization was considered in the model, as the hot, dry, and windy conditions are optimal for ammonia volatilization. In addition, NH_3 emissions can increase exponentially with increasing N additions (Jiang et al. 2017). Generally, plants exhibited a preference for NH_4^+ when the soil N pool was strongly dominated by NH_4^+ or together NH_4^+ and NO_3^- in a natural situation (Nordin et al. 2001). This was the reason why the NH_4^+-N taken up by plants was also considered in the model.

The N transformations among the pools are described by the following equations:

$$\frac{dC_{NH_4^+}}{dt} = -f_{hd}(WFP,T)k_1C_{NH_4^+} - f_{an}(WFP,T)k_4C_{NH_4^+} - f_{nd}(WFP,T)k_6C_{NH_4^+} - f_{av}(WFP,T)k_8C_{NH_4^+} - k_9C_{NH_4^+} \quad (1)$$

$$\frac{dC_{NO_3^-}}{dt} = +f_{hd}(WFP,T)k_1C_{NH_4^+} - f_{hd}(WFP,T)k_2C_{NO_3^-} - \text{Rainfall}k_{10}C_{NO_3^-} \quad (2)$$

$$\frac{dC_{N_2Osoil-1}}{dt} = +f_{hd}(WFP,T)k_2C_{NO_3^-} + f_{nd}(WFP,T)k_6C_{NH_4^+} - f_{hd}(WFP,T)k_3C_{N_2Osoil-1} - f_{hd}(WFP,T)k_7C_{N_2Osoil-1} \quad (3)$$

$$\frac{dC_{N_2Osoil-2}}{dt} = +f_{an}(WFP,T)k_4C_{NH_4^+} - f_{an}(WFP,T)k_5C_{N_2Osoil-2} \quad (4)$$

$$\frac{dC_{N_2Oair}}{dt} = +f_{hd}(WFP,T)k_7C_{N_2Osoil-1} + f_{an}(WFP,T)k_5C_{N_2Osoil-2} \quad (5)$$

where C denotes the concentration of each pool mentioned above (also reported in Fig. S1), k is the first-order kinetic parameter of each pathway, and Rainfall is daily rainfall amount during the experiment (Fig. 1a). $f_{hd}(WFP, T)$, $f_{an}(WFP, T)$, $f_{nd}(WFP, T)$, and $f_{av}(WFP, T)$ represent the effects of soil water and temperature on four N processes (heterotrophic denitrification, autotrophic nitrification, nitrifier denitrification, and ammonium volatilization), which can be expressed as the product of the response functions of soil water and soil temperature:

$$f(WFP,T) = f(WFP)\cdot f(T)$$

The influence of soil temperature on each transformation process was modeled by Ratkowsky (Jansson 2001).

$$f(T) = \begin{cases} 0 & T < t_{min} \\ \left(\frac{T - t_{min}}{t_{max} - t_{min}}\right)^2 & t_{min} < T < t_{max} \\ 1 & T > t_{max} \end{cases}$$

where the values of t_{min} and t_{max} are unknown and to be estimated together with other kinetic parameters. The response of different transformation processes to soil water was introduced by piecewise linear equations (Fig. S2) (Müller 1999):

$$f(WFP) = \begin{cases} 0 & WFP \le WFP_1 \\ a_+ + b_+ \cdot WFP & WFP_1 \le WFP \le WFP_2 \\ 1 & WFP_2 \le WFP \le WFP_3 \\ a_- + b_- \cdot WFP & WFP_3 \le WFP \le WFP_4 \\ 0 & WFP \ge WFP_4 \end{cases}$$

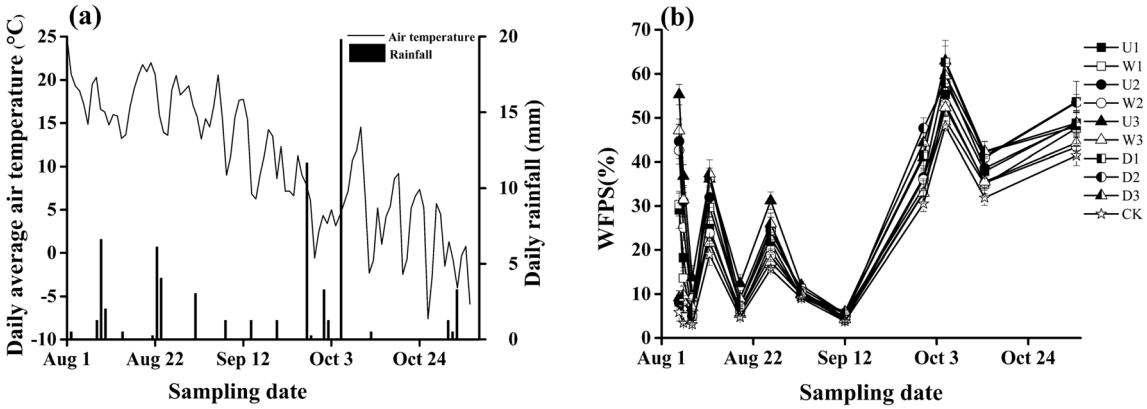

Fig. 1 **a** Daily average air temperature (°C) and rainfall (mm). **b** Water-filled pore space (WFPS) of soils after treatments throughout the study period. CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine; W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

As listed in Table S2, all the unknown parameters were simultaneously estimated by Markov Chain Monte Carlo (MCMC), which has been shown to be more effective than traditional least-square fitting methods (Müller et al. 2007). To generate samples from posterior distributions efficiently, we used the recently developed DiffeRential Evolution Adaptive Metropolis algorithm ($DREAM_{(zs)}$). The details of $DREAM_{(zs)}$ can be found in Vrugt (2016). To guarantee the accuracy, Gelman-Rubin $\hat{R}$ scale reduction factor was applied to judge the convergence of $DREAM_{(zs)}$ (Gelman and Rubin 1992). The ODE solver (ode15s) was employed to build the model structure.

Statistical analysis

All statistical analyses were performed using SPSS version 20, and one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to check for the differences of soil mineral N contents, N_2O flux, and functional gene abundance between treatments. Relationships among dynamics of abundances of functional genes, N_2O emissions, soil mineral N concentrations, and soil WFPS were determined using Pearson's correlation coefficients (Hall 2015). It was considered significant for $P < 0.05$. All the figures were prepared by Origin (Origin Pro 9.0 for Windows).

Results

Environmental conditions

Daily average air temperature was high at the start of the experiment in August and gradually decreased with time, varying from a high of 25 °C to a low of − 8 °C during the experimental period (Fig. 1a). This temperature dynamics corresponded to the mean annual temperature decline in autumn in this region. The rainfall was 75.2 mm during the experimental period, which was slightly lower than the annual average rainfall during this period for the region (100 mm). The soil moisture content (water-filled pore space, WFPS; Fig. 1b) fluctuated with time as a result of rainfall events and varied from a low of 3.10% to a high of 62.97% during the experiment period. The WFPS was below 50% for most of the experimental period.

Total N_2O emissions

Results from our study showed that total N_2O emissions (Fig. 2) were very low under the dung and water treatments, ranging from 0.005 to 0.02 kg ha^{-1}, and there were no significant differences between the different water or dung rates. The total N_2O emissions in the urine treatments were significantly higher than those in the dung and water treatments, ranging from 0.12 to 0.78 kg ha^{-1} and increased significantly with increasing urine application rates. The percentages of N applied that was emitted as N_2O-N under the U1, U2, and U3 treatments were 0.05, 0.07, and 0.1%, respectively, and ranged from 0.0002 to 0.001% under the dung treatments, respectively.

The contribution of each pathway to N_2O emissions by a N transformation model

To quantify the complex N transformations in the grazed grassland soil, we used a complex process-based model (Fig. S1) that considered six pools and ten N transformation processes based on the microbial pathways for N_2O formation in soil (Saggar et al. 2004; Cardenas et al. 2007; Loick et al. 2017). Three predominant biotransformation processes, including autotrophic nitrification, heterotrophic denitrification, and nitrifier denitrification for N_2O production in the soils, were considered. As mineral

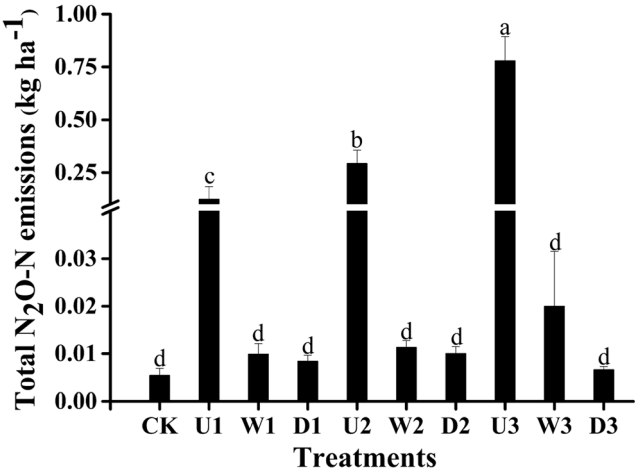

Fig. 2 Total N_2O emissions as affected by treatments. The vertical bars indicate the standard error of the mean (S.E.M.). CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine; W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

nitrogen (NH_4^+-N and NO_3^--N) contents and N_2O emissions were slightly affected by water and dung treatments compared with the CK treatment, mathematical models were applied under the three urine treatments (U1, U2, and U3) and the high rates of water (W3) and dung (D3), plus CK. To evaluate the goodness of model-fitting, we performed the evolution of statistic (Fig. S5), quantile-quantile (QQ) plots (Fig. S6), and compared observed and simulated data (Fig. S7) in U1, U2, U3, W3, D3, and CK treatments. The evolution of the Gelman-Rubin statistic was used to monitor the convergence of the sampled Markov chains. Convergence was reached for all the chains, i.e., the statistic went below the convergence threshold (the dotted black line) (Fig. S5). The normality assumption of the model residual used in the Bayesian framework was assessed via QQ plots (Fig. S6). Although some outlier values were evident in the tails of the distribution, especially in Fig. S6a, the model residuals were overall modeled appropriately. After the convergence, the maximum-a-posterior (MAP) estimations of parameters, which corresponded to the minimal RMSE (root-mean-square error), were chosen to obtain the simulated data. The observed and simulated data for six treatments are shown in Fig. S7. In general, the simulated values fit the observed data well. Although some data of hourly N_2O-N flux were underestimated by the model, the trends of simulated data were consistent with those of observed data. This confirmed the validity of the proposed model. The estimated model parameters showed that the three urine treatments led to similar results, and all the parameters in the present model were within a reasonable range (Table S2; Figs. S8 and S9), according to Müller et al. (2004).

The water-treated soils shared similar N transformations with the control soils (Fig. 3). Autotrophic nitrification dominated the generation of N_2O emissions but its fraction decreased gradually while nitrifier denitrification played a more important role in N_2O emissions with time in the U1 and U2 samples (Fig. 3). In the U3 soils, heterotrophic denitrification accounted for a significant share in generation of N_2O at the early stage, while nitrifier denitrification was the dominant pathway to generate N_2O at the later stage (Fig. 3). The D3 samples showed similar N transformation with the U3 samples, with less heterotrophic denitrification (Fig. 3).

Abundance of nitrifiers' and denitrifiers' functional genes

The abundance of nitrifiers and denitrifiers was evaluated by quantitative PCR of bacterial and archaeal *amoA* genes, *nirS*, *nirK*, and *nosZ genes* in soil samples for each treatment along the 91-day field study, respectively (Figs. 4 and 5). The abundances of the AOA *amoA* gene in the soil were generally higher (3.66×10^9–2.18×10^{10} copies g^{-1} soil) than those of AOB (2.25×10^8–5.22×10^9 copies g^{-1}) (Fig. 4). The application of urine-N or water alone or sheep dung did not lead to major changes in AOA *amoA* gene copy numbers (Fig. 4a–c). In stark contrast, the AOB *amoA* gene abundance increased significantly following the application of the sheep excreta and water ($P < 0.05$) compared with the CK treatment (Fig. 4d–f).

There was a stronger linear relationship between the NO_3^--N concentration in the soil and the AOB *amoA* gene abundance ($r = 0.617$, $P < 0.001$) than with the AOA *amoA* gene abundance ($r = 0.335$, $P < 0.05$) (Table 1). In addition, a significant relationship was found between the N_2O flux and the AOB abundance ($r = 0.373$, $P < 0.001$), but not to that of AOA (Table 1).

Of all the denitrifying functional genes studied here, *nirK* was the only gene whose abundance increased significantly following the urine-N application at the high rate (triple urine application) (Fig. 5d). The abundance of all other denitrifying functional genes, including *nirS* and *nosZ*, remained stable following the urine, dung or water treatments (Fig. 5). In addition, the abundance of the *nirK* gene was significantly related to the NO_3^--N concentration in the soil ($r = 0.380$, $P = 0.007$) and to the N_2O emissions ($r = 0.614$, $P < 0.001$) (Table 1).

Composition of soil microbial communities

As the abundances of archaeal and bacterial *amoA* genes and *nirK*-type denitrifying genes were significantly related to soil NO_3^--N content and N_2O emissions (Table 1), we conducted high-throughput sequencing of 16S rRNA genes using the

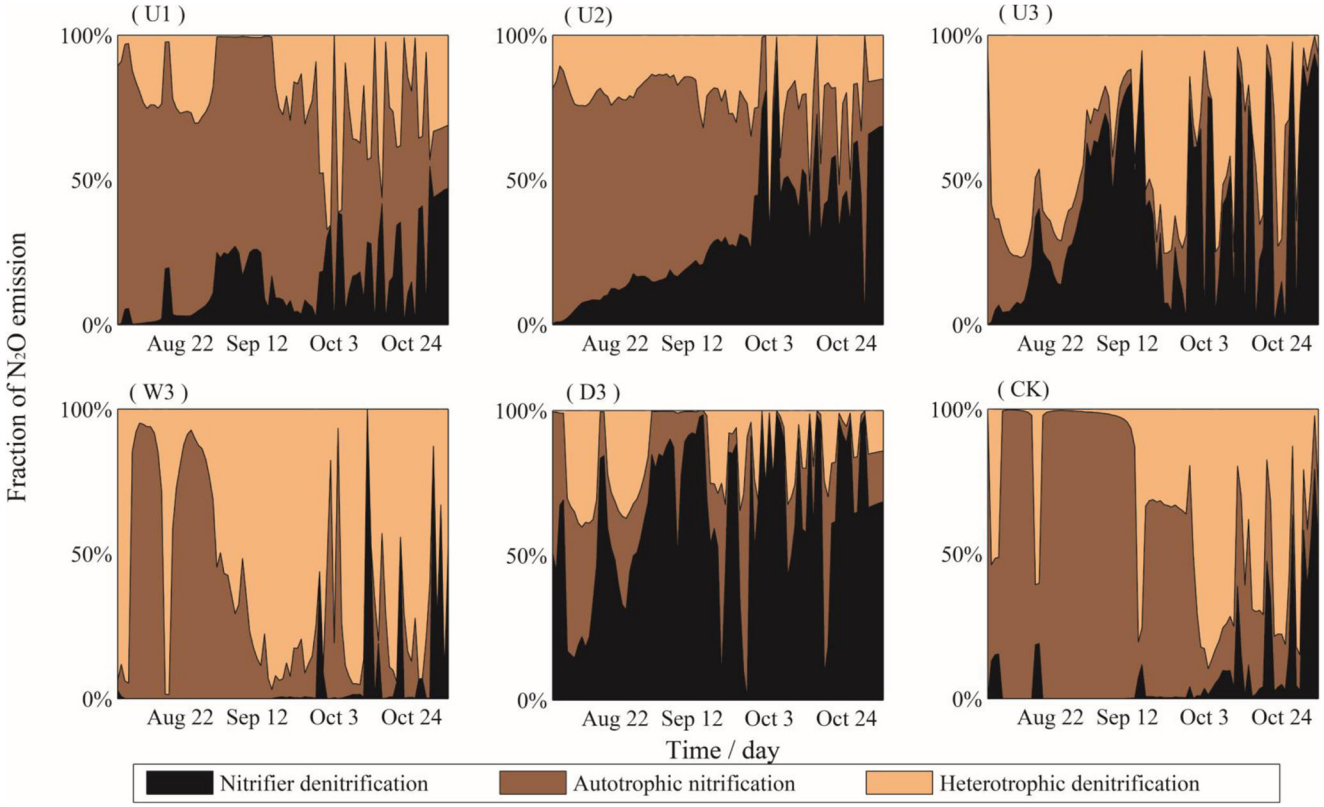

Fig. 3 Fractions of pathway specific N_2O emissions over time as affected by U1, U2, U3, W3, D3 and CK treatments. The parameters of the model were estimated by DiffeRential Evolution Adaptive Metropolis algorithm ($DREAM_{(zs)}$), which was a tool for Bayesian parameter inference. The rate of each transformation was obtained by maximizing the likelihood. CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine. W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

universal primers 515F-907R for *amoA* genes and cloning analysis for *nirK* genes. The primers and PCR conditions are detailed in Table S1. The samples used to carry out community analysis were sampled on September 2, 2014, under the three urine treatments (U1, U2, and U3) and the high rates of water (W3) and dung (D3), plus control (CK). Significant changes were revealed in the nitrifier and denitrifier functional gene abundances and in the dynamics of soil mineral N contents and hourly N_2O fluxes at the sampling dates during the 3-month field experiment under these treatments (Figs. 4 and 5; Figs. S3 and S4).

Approximately 670,269 high-quality sequence reads were obtained (Table S3). The relative abundance of bacterial 16S rRNA genes ranged from 93.5 to 99.8%, and this was much higher than that of archaeal 16S rRNA genes, which ranged from 0.2 to 6.5%. The 16S rRNA genes affiliated with AOA and AOB were selected for phylogenetic analysis from the total MiSeq reads (Fig. S10a and S10b). All the archaeal 16S rRNA genes were affiliated with the soil group I.1b lineage, containing six distinct OTUs within five clusters: 29i4 cluster, 29i4 associated cluster, 54d9 cluster, *N. viennensis* associated cluster, and *N. viennensis* cluster (Fig. S10a). With respect to AOB, the bacterial 16S rRNA genes were classified into five OTUs, which fell into three clusters: *Nitrosomonas oligotropha*, *Nitrosospira* cluster 3, and *Nitrosococcus* (Fig. S10b). Clone library construction demonstrated that the *nirK* genes were clustered into 58 OTUs, three of which were unclassified (Fig. S10c). The other OTUs were phylogenetically related to Bradyrhizobium, Paracoccus, Devosia, Mesorhizobium, Pseudomonas, and Rhodobacter.

In order to understand the dynamics of community distribution of functional genes, we also analyzed the proportional changes of these genes (Fig. 6). The application of different urine-N rates significantly changed the proportions of AOA, AOB, and *nirK* phylotypes. The *N. viennensis* associated cluster dominated the AOA community in the typical steppe grassland soils studied here (Fig. 6a). The U3 treatment resulted in the absence of the 54d9 cluster. The majority of AOB, up to 42.9%, was affiliated with *Nm. Oligotropha* and another 42.9% was affiliated with *Nitrosococcus*. The proportion of cluster 3 increased, while that of *Nm. oligotropha* decreased with increasing urine-N rates. The W3 treatment led to a significant increase in *Nm. oligotropha* and a decrease of cluster 3. The D3 treatment, however, increased the proportion of

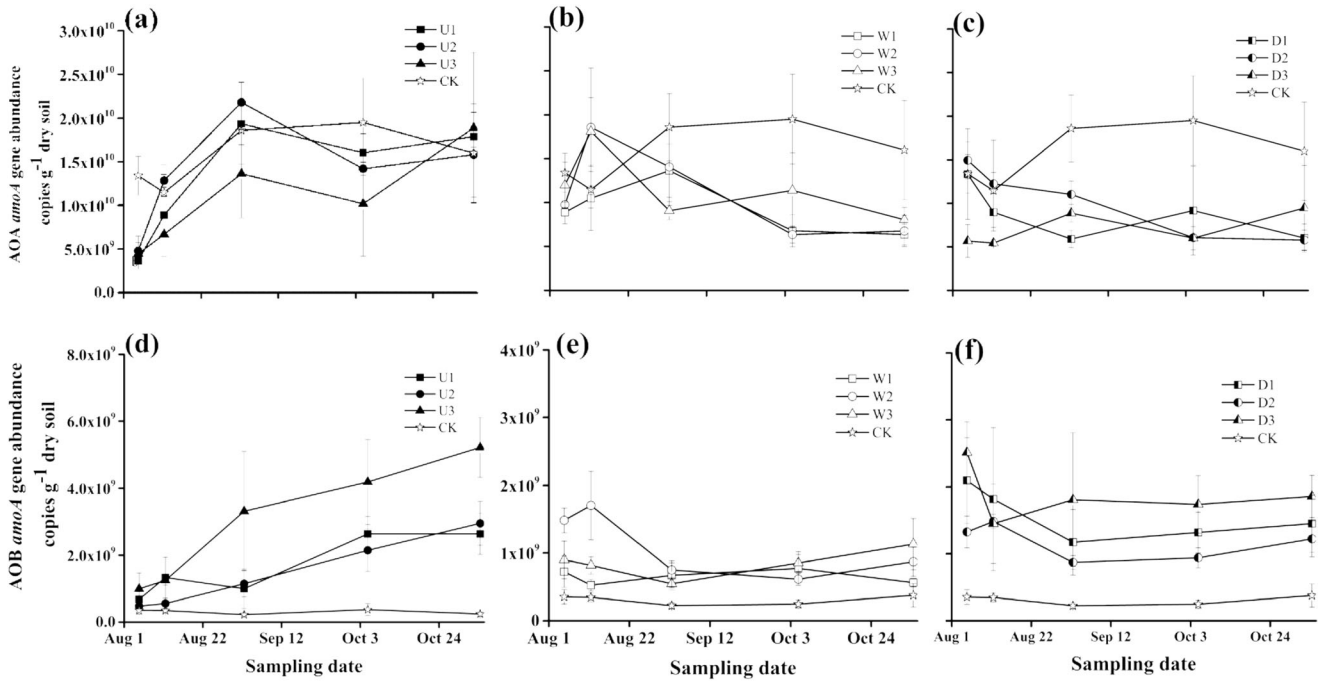

Fig. 4 AOA *amoA* gene copy numbers in different soils as affected by **a** urine treatments, **b** water treatments, and **c** dung treatments. AOB *amoA* gene copy numbers in different soils as affected by **d** urine treatments, **e** water treatment, and **f** dung treatment. The vertical bars indicate the standard error of the mean (S.E.M.). CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine; W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

Nitrosococcus and cluster 3 and decreased that of *Nm. oligotropha.* As for *nirK* (Fig. 6c), the relative abundance of the order Rhizobiales significantly increased with increasing urine-N rates, while that of Rhodobacterales decreased. The U3 treatment led to the absent of the order Rhodobacterales. The order Pseudomonadales was exclusively found under the D3 treatment.

Discussion

The percentage of N applied emitted as N_2O-N recorded, when a complete emissions envelope was obtained, was equivalent to the emission factor (EF) in the U1 treatment (Fig. S4a). Therefore, the 0.05% EF in the U1 treatment recorded in this study for a single sheep urine deposition was much lower than the international default value of 2% (IPCC 2000) and was much lower than those reported in a number of other studies for pasture soils (Saggar et al. 2007; Barneze et al. 2015) or sheep patch soils (Oenema et al. 1997; de Klein et al. 2003; van Groenigen et al. 2005; Selbie et al. 2014). Likewise, the 0.0013 to 0.002% values measured here for sheep dung were also much lower than those reported in other countries (Oenema et al. 1997; Cardenas et al. 2016). The low N_2O emissions from the urine and dung in this study were probably due mainly to the very dry soil conditions (Dobbie and Smith 2003; Liu et al. 2017; Wang et al. 2017). The soil WFPS was mostly between 10 and 30% for large parts of the experimental period and rarely exceeded 50% (Fig. 1b). Such conditions are unfavorable for heterotrophic denitrification to occur to produce N_2O from the soil. This is supported from results of process-based modeling analysis (Fig. 3) and the lack of growth of most of the denitrifiers that were measured in this study (Fig. 5). Denitrification can also be driven by microbial oxygen consumption and may occur at low WFPS levels in high C pasture soils (Petersen et al. 2013; Balaine et al. 2016; Friedl et al. 2016). However, the soil organic C content was also very low (15.84 g kg^{-1}) in the present study. Moreover, the significant relationship between N_2O emissions and *nirK*/*nosZ* ratios ($r = 0.572$, $P < 0.001$) further suggests that N_2O consumption was a possible explanation for the low N_2O emissions (Table 1). Another interesting result revealed by the present study was the little impact of fresh dung on the dynamics of N_2O emissions while previous studies have reported markedly increased N_2O emissions following dung application (Ma et al. 2006; Lin et al. 2009). This discrepancy may have been caused by the very dry soil conditions of this study where the dung was not decomposed significantly. Further research is required to understand effects of environmental conditions on sheep dung decomposition and effects on N_2O emissions in low fertility and dry grassland soils.

Although N_2O emissions from both the urine and dung treatments were extremely low, the process-based model

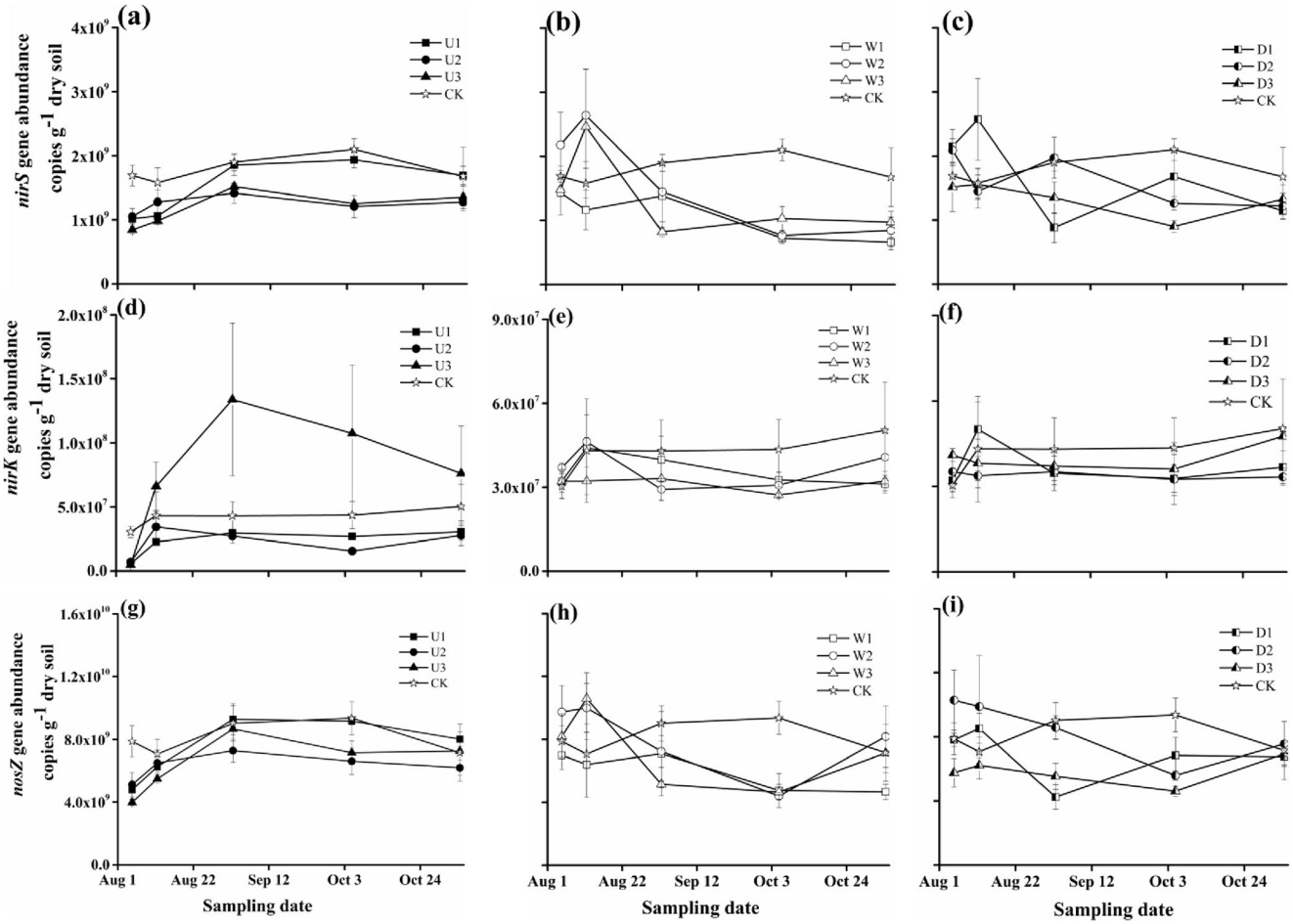

Fig. 5 *NirS* gene copy numbers in different soils as affected by **a** urine treatments, **b** water treatments, and **c** dung treatments. *NirK* gene copy numbers in different soils as affected by **d** urine treatments, **e** water treatments, and **f** dung treatments. *NosZ* gene copy numbers in different soils as affected by **g** urine treatments, **h** water treatments, and **i** dung treatments. The vertical bars indicate the standard error of the mean (S.E.M.). CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine; W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

indicated that the contribution of each pathway to N_2O emissions varied with urine and dung treatments along the sampling time (Fig. 3). The importance of autotrophic nitrification under the U1 and U2 treatments at the early stage was ascribed to the greater advantages of the nitrifiers than denitrifiers after urine addition (Lu et al. 2012; Lu and Jia 2013), as the AOB populations flourished following high N inputs (Fig. 4d) (Di et al. 2009). In contrast, heterotrophic denitrification had a significant share in the generation of N_2O in the U3 samples at the early stage (Fig. 3). This phenomenon might be ascribed to the rapid growth of heterotrophic microorganisms, which was stimulated by the large addition of urea, leading to anoxic conditions because of consumption of soil oxygen (Bodelier et al. 1996), although the soil WFPS was fluctuating around 30% (Fig. 1b). The evident growth of *nirK*-type denitrifier in the U3 samples at the early stage further indicated the importance of heterotrophic denitrification for the generation of N_2O (Fig. 5d). Although nitrifier denitrification could be neglected in the W3 and CK soils, it increased with time in the U1 and U2 treated soils, gradually dominating the N_2O emissions in the U3 and D3 treated soils (Fig. 3), which corresponded well with the increases in the abundance of bacterial *amoA* genes (Hink et al. 2017, 2018), as shown by quantitative PCR (Fig. 4d, f). The results agreed with the findings from a study conducted in a dairy farm in the Netherlands, which showed a larger contribution of nitrifier denitrification to the total N_2O production after the application of artificial urine (Wrage et al. 2004). Wrage et al. (2004) and Zhu et al. (2013) suggested that larger NO_2^--N concentrations following urine application would stimulate nitrifier denitrification. Di et al. (2014) previously reported that the dynamics of *nirK* gene abundance resembled that of AOB *amoA* gene abundance. Some AOB populations contain the *nirK* gene, and therefore, they were able to grow under both aerobic and anaerobic soil conditions performing nitrifier denitrification. Stein (2011) also proposed that AOB, *nirK*, and *norB* were the

Table 1 Pearson correlations (*R* values) between microbial population abundance and abiotic factors

	WFPS (%)	Exchangeable NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	N_2O (μg m^{-2} h^{-1})	AOA (copies g^{-1} d.w.s.)	AOB (copies g^{-1} d.w.s.)	*nirS* (copies g^{-1} d.w.s.)	*nirK* (copies g^{-1} d.w.s.)	*nosZ* (copies g^{-1} d.w.s.)	AOA/AOB	*nirK*/*nosZ*
WFPS (%)	–	NS	NS	NS	**0.044**[b]	NS	**0.020**	NS	**0.069**	**0.045**	NS
Exchangeable NH_4^+-N (mg kg^{-1})	− 0.028a	–	NS	NS	**0.038**	NS	NS	**0.032**	**0.012**	NS	NS
NO_3^--N (mg kg^{-1})	0.196	− 0.031	–	**< 0.001**	**0.017**	**< 0.001**	NS	**0.007**	NS	NS	**0.01**
N_2O (μg m^{-2} h^{-1})	0.010	0.171	**0.554**	–	NS	**< 0.001**	NS	**< 0.001**	NS	NS	**< 0.001**
AOA (copies g^{-1} d.w.s.)	**0.286**	**− 0.294**	**0.335**	0.107	–	NS	**< 0.001**	NS	**< 0.001**	**< 0.001**	NS
AOB (copies g^{-1} d.w.s.)	0.227	− 0.093	**0.617**	**0.373**	0.187	–	NS	**< 0.001**	NS	**< 0.001**	**0.001**
nirS (copies g^{-1} d.w.s.)	**0.329**	− 0.237	0.019	− 0.131	**0.597**	0.101	–	NS	**< 0.001**	**0.053**	NS
nirK (copies g^{-1} d.w.s.)	0.056	− 0.305	**0.380**	**0.614**	0.179	**0.542**	0.134	–	**0.097**	NS	**< 0.001**
nosZ (copies g^{-1} d.w.s.)	**0.260**	− 0.275	0.124	− 0.025	**0.663**	0.110	**0.819**	**0.237**	–	**0.049**	NS
AOA/AOB	**0.284**	− 0.116	− 0.214	− 0.133	**0.479**	**− 0.489**	**0.275**	− 0.005	**0.280**	–	NS
nirK/*nosZ*	0.048	− 0.232	**0.361**	**0.572**	− 0.079	**0.452**	− 0.188	**0.884**	− 0.171	− 0.098	–

Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) correlations are indicated in bold

NS not significant

[a] *R* values (left-bottom half part of the table)

[b] *P* values for the same correlations (right-top half of the table)

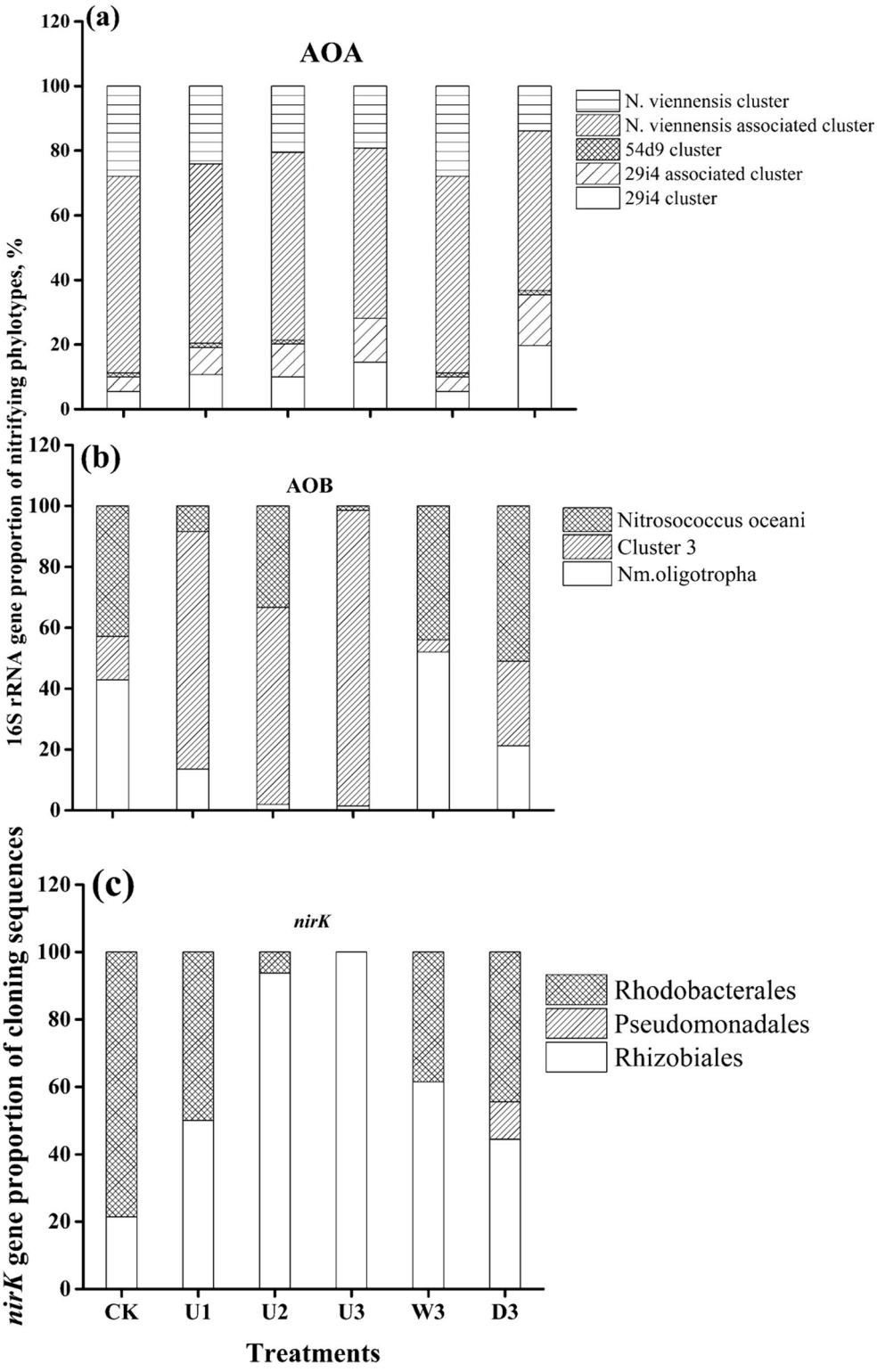

Fig. 6 Proportional changes of **a** AOA, **b** AOB, and **c** *nirK* phylotypes in response to excreta treatments. The changes in AOA and AOB composition were analyzed by target 16S rRNA genes in each treatment. The changes in *nirK* composition were analyzed by cloning analysis. CK, control, nothing was applied; U1, 0.4375 L synthetic sheep urine; U2, 0.875 L synthetic sheep urine; U3, 1.3125 L synthetic sheep urine; W1, 0.4375 L water; W2, 0.875 L water; W3, 1.3125 L water; D1, 300 g fresh dung; D2, 600 g fresh dung; D3, 900 g fresh dung. The urine and dung application rates corresponded to about 218, 436, 654, 233, 465, and 698 kg N ha^{-1}, respectively

sole nitrifiers and reductases, respectively, in the nitrifier denitrification pathway. It is therefore not surprising that N_2O emissions were positively correlated to the abundance of both AOB ($r = 0.373$, $P < 0.001$) and *nirK* genes ($r = 0.614$, $P < 0.001$), respectively (Table 1). Given the above, we speculated that nitrifier denitrification dominated N_2O production in this grazed dry and low fertility grassland soil. Nitrifier denitrification in natural ecosystems has not received much attention (Butterbach-Bahl et al. 2013; Shcherbak et al. 2014; Stieglmeier et al. 2014; Shi et al. 2017). Our results suggested

that nitrifier denitrification, a previously overlooked process, was indeed an important pathway for N_2O emissions in this typical steppe grassland soil. The validity of the mathematical model was confirmed by a good fit to the experimental data, as shown by the observed and simulated data of NH_4^+-N contents, NO_3^--N contents, and N_2O fluxes (Fig. S7) in the U1, U2, U3, W3, D3, and CK treatments. The statistic (Fig. S5) and quantile-quantile (QQ) plots (Fig. S6) confirmed the convergence and effectiveness of the estimated results. In addition, the abundance dynamics of N cycling-related functional genes (Figs. 4 and 5) were also evaluated in the present experiment to further validate the used model. Although the presence of genes does not necessarily mean active expression, the dynamic changes of the functional genes under different treatments along with time might reveal functional activity. It should be pointed out that, although DREAM(zs) is a powerful tool to simultaneously estimate multiple rates from limited data, uncertainties always exist in the estimated results. It is well recognized that using isotopes or inhibitors helps to distinguish the relative contributions of microbial pathways to N_2O yields (Shi et al. 2017). From a point view of Bayesian parameter inference, the use of isotopes or inhibitors leads to less uncertainties (i.e., more confidence) in the estimation of results. Further research combining isotopes or inhibitors with process-based modeling introduced in the present study would be useful.

Previous studies of high fertility dairy pasture soils have shown that ammonia oxidation is mainly performed by AOB in the high N status urine patch soil, and AOA population abundance is sometimes inhibited by the high N inputs in the form of animal urine-N (e.g., Di et al. 2009, 2010, 2014). In addition, AOA played a predominated role in acid soil nitrification while AOB tended to dominate nitrification in alkaline soils (Shi Gubry-Rangin et al. 2010; Zhang et al. 2012; Hu et al. 2014). However, results from this study showed that both AOB and AOA were positively correlated to the NO_3^--N concentrations in the soil (Table 1), which suggested that both AOA and AOB played a role in ammonia oxidation in this low fertility and slightly alkaline sheep grassland soil. In addition, the community compositions of AOB and AOA found in this low fertility sheep pasture soil were also different from those reported in the high fertility pasture soils. For example, the dominant AOA in this sheep pasture soil after urine-N application was *N. viennensis* and associated cluster, whereas those in the grassland soils established at Hillsborough, UK, were dominantly by fosmid 29i4-like AOA (Zhou et al. 2015). This discrepancy might be because that *N. viennensis* cluster was urease-positive and would grow optimally in slightly alkaline typical steppe grassland soils (Tourna et al. 2011). Similarly, the dominant AOB found in this sheep pasture soil were *Nitrosococcus* and *Nitrosospira,* whereas those found in the UK grassland soils were *Nitrosospira* cluster 3-like AOB (Zhou et al. 2015). In fact, both *Nitrosococcus* and *Nitrosospira* species of AOB were shown to contain *nirK* and *norB* genes and could carry out nitrifier denitrification (Norton et al. 2008; Stein 2011; Klotz et al. 2006). These results further suggested the occurrence of nitrifier denitrification in the typical steppe grassland soils. Based on the *nirK* clone library analysis (Fig. 6c; Fig. S10c), most of *nirK*-harboring denitrifiers were related to the *nirK* from Rhizobiales and Rhodobacterales, similar to findings from previous studies (Bremer et al. 2007; Saito et al. 2008; Pan et al. 2016). It was also worth mentioning that the relative abundance of the order Rhizobiales increased with increasing urine-N rates, while that of Rhodobacterales changed in opposite direction, decreasing with the high rate of urine (U3) treatment (Fig. 6c) but occupied high proportions in the dung and water treatments. The changing trend of the relative abundance of the order Rhizobiales was similar with that of nitrifier denitrification pathway in the urine patches. The predomination of nitrifier denitrification for the N_2O emissions in the U3 indicated that *nirK*-type denitrifiers of Rhizobiales were probably involved in nitrifier denitrification.

Conclusions

Results from this study showed that in this dry and low fertility sheep grassland soil, the N_2O emissions from urine patches were significantly higher than those from dung; both were much lower than previously predicted. The microbial pathways of N_2O production varied widely with sheep excreta. Nitrifier denitrification, a traditionally overlooked process, was indeed a process producing significant N_2O occurred in soils following inputs of urea. Nitrification dominated N_2O emissions in environments high in oxygen while heterotrophic denitrification in micro-aerobic conditions. These results help to understand the complex biotransformation processes of N in the dry and low-fertility steppe grassland and are important in developing future management practices for the mitigation of N_2O emissions in grazed grasslands.

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