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Effects of pasture management on soil fertility and microbial communities in the semi-arid grasslands of Inner Mongolia

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Abstract
Purpose Grasslands are an important ecosystem covering about a quarter of the earth's surface. Different management practices of grassland ecosystems can have a major impact on the sustainability of these ecosystems. The objective of this study was to determine the impact of different pasture management practices on soil properties and microbial communities in the semi-arid grassland ecosystem in Inner Mongolia.
Materials and methods Long-term experimental plots were established in the semi-arid grasslands of Inner Mongolia to study the effect of different grazing practices on soil properties and microbial communities. The treatments included (1) enclosure from grazing since 1983 (E83), (2) enclosure from grazing since 1996 (E96), and (3) continuous free grazing (FG). We collected the soil samples from these treatments to study soil properties and microbial biomass abundance and diversity. An incubation study was also conducted using soils from E96 and FG treatments to determine the growth responses of ammonia oxidizers to urea addition.
Results and discussion Soil organic matter and total N increased when the grassland was enclosed from grazing, but soil fertility did not increase further with continued enclosure extending from 1996 to 1983. Enclosure also increased microbial biomass but did not significantly affect the microbial diversity. Both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) grew when supplied with urea-N, but the growth rate was higher in the soil from FG than in the soils from enclosed areas. The phospholipid fatty acids (PLFAs) of bacteria i15:0, 16:1 ω7c, 16:1 ω5c, 16:0, 18:1 ω7c, and actinomycetes 10-Me-16:0 used the most of the ^{13}C-urea in both the E83 and FG soils. There was higher incorporation of ^{13}C in PLFA 16:0 in the E83 soil after 3 and 7 days incubation, compared with the FG soil, suggesting higher metabolic activity in the E83 soil than the soil from the FG treatment. Most of the effects by the different pasture management practices were confined to the surface soil (0–20 cm), and there was minimal effect in the subsoils (below 20 cm).
Conclusions These results suggest that enclosure of grassland from grazing not only affects soil fertility but also microbial biomass and ammonia-oxidizing populations. Microbial communities are sensitive to pasture management changes, and these have implications to nutrient cycling and management in these grassland ecosystems.

Keywords Pasture management · Microbial community structure · Stable isotope probing

1 Introduction

Grasslands cover approximately 25.4 % of the total land area of the earth (Watson et al. 2000). At present, China's total grassland area is approximately 400 million hectares, accounting for 11.7 % of the earth's total grassland area and 41.7 % of China's land area (Liao and Jia 1996). Grassland is an important terrestrial ecosystem and one of the most widely distributed ecosystems in the world. In terms of climate change, grassland has a significant impact on global carbon source/sink dynamics and carbon cycling (Cheng et al. 2012).

Overgrazing has not only decreased the productivity of grasslands but also resulted in a dramatic decline in the quality

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of forage and plant diversity, thus having a negative impact on the sustainability of grassland (Li et al. 1994). This is manifested not only by a loss of biomass but also by the invasion of undesirable plant species (Karnieli et al. 2013). The original dominant species in Inner Mongolia grassland were *Leymus chinensis* and *Stipa grandis*, but were gradually dominated by *Artemisia frigida*, due to overgrazing (Li and Wang 1999). Overgrazing also has adverse impacts on soil physical, chemical, and biological properties (Li and Zhao 2005).

Large areas of grasslands in China have been severely degraded due to overgrazing (Chen 1990). In order to prevent further degradation and restore degraded grasslands, areas of degraded land are fenced off from grazing for periods of time. During this recovery phase of degraded grasslands, pasture composition and microbial communities undergo significant changes. For example, Shan et al. (2008) found that the productivity and species diversity of severely degraded grassland increased after 14 years of enclosure but decreased after 14 years of enclosure. The soil nutrient contents also increased significantly during the restoration phase (Shan et al. 2009).

Our knowledge and understanding of microbial community composition and abundance in relation to the different phases of grasslands management is limited. This limits our ability to predict and manage grasslands under a changing climate. It also limits our understanding on how grasslands will impact on and respond to climate change. Therefore, further studies are required to determine changes in microbial communities in grasslands as affected by animal grazing and different restoration phases of grassland management.

The objective of this study was to determine the effect of different pasture management on soil nutrition availability and microbial communities in the semi-arid grasslands of Inner Mongolia.

2 Materials and methods

2.1 Study site

The research site, Baiyinxile grassland, is situated in Eastern Inner Mongolia (N 43° 33′ 12″–43° 33′ 35″, E 116° 42′ 26″–116° 42′ 31″; 1000–1500 m above sea level). The region has a temperate semi-arid continental climate with an annual average temperature of −0.1 °C, annual mean precipitation of 280–350 mm, and annual evaporation 4–5 times that of the precipitation. From March to May, the weather is windy and the monthly average wind speed is 4.9 m s^{-1}. Frost-free period is about 90 days. The soil is chestnut soil (Mollisols), on gently sloping terrain. *L. chinensis* and *S. grandis* are typical original pasture species in the region, but because of overgrazing, other invading species, including *Agropyron michnoi*, *A. frigida*, *Potentilla acaulis*, and *Cleistogenes squarrosa* have become dominant species.

2.2 Field experimental design

Three pasture management practices (two different periods of enclosure and one without enclosure with continuous grazing) were studied in terms of their impact on soil properties and microbial communities. One treatment of non-grazed enclosure plots had been enclosed free from grazing since 1983 (E83), and the plot size was 600 × 400 m; another non-grazed enclosure treatment had been enclosed since 1996 (E96), and the plot size was 80 × 400 m. The non-enclosed plots with continuous grazing was used as a control treatment where nomads were allowed to graze throughout the year since 1983 (FG), and the plot size was 600 × 50 m. The plots were arranged in a random design in the field.

Five soil cores at three different depths (0–20, 20–40, and 40–60 cm) were collected randomly from three plots for each treatment in October 2013, and they were mixed for each depth to form one composite sample for each plot. The soil samples were placed in sterile plastic bags, sealed and transported to the laboratory on ice. One part was sieved through 2.0 mm for analysis of chemical properties according to the methods described by Sparks et al. (1996), subsamples were stored at −80 °C for DNA extraction and molecular analysis, and another part was incubated by stable isotope probing to determine differences of soil microbial structure community immediately after arrival to the laboratory.

2.3 Soil microbial biomass analysis

The fumigation extraction method was used to determine soil microbial biomass C (MBC) and soil microbial biomass N (MBN) (Brookes et al. 1985; Vance et al. 1987). Briefly, 10 g (oven-dry equivalent) was weighed into 100 mL bottles. Three unfumigated samples from each treatment were immediately extracted with 50 mL 1.0 M K_2SO_4 on a rotary shaker at 220 rpm for 30 min and then filtered through medium-speed qualitative filter paper (Chantigny et al. 2008). The remaining samples (triplicates) were fumigated with alcohol-free chloroform for 24 h at 25 °C. Excess chloroform was removed by repeated evacuation, and then the samples were extracted and filtered as described above. The filtrates were stored at −20 °C prior to analysis for MBC and MBN.

2.4 Stable isotope probing microcosms

Soil microcosms were constructed by adding approximately 50 g (dry weight basis) of soil from the E83 and FG (0–20 cm) treatments to 100 mL plastic cans. The plastic cans were covered with lids which had a hole (the diameter is 1 cm) pierced on the top. The soils were maintained at 60 % water holding capacity in the soil microcosms for 1 week prior to use. Following this pre-incubation period, soils were subjected to one of three treatments: CK (0), ^{13}C (200 mg kg^{-1} ^{13}C- urea-N),

^{12}C (200 mg kg^{-1} ^{12}C- urea-N). Either 2.72 mL of sterile deionized water (control) or 2.72 mL of a solution containing 20 mg mL^{-1} urea-N labeled or unlabeled (equivalent to additions of 200 mg kg^{-1} urea-N) was added to the soils. The labeled urea applied had a ^{13}C enrichment at 99 atom % (Sigma-Aldrich, USA). This gave a total of six treatments (CK-E83, ^{13}C-E83, ^{12}C-E83 and CK-FG, ^{13}C-FG, ^{12}C-FG) with three replicates. The microcosms were randomly arranged and incubated at 4 °C in the dark. Deionized water was added every day to maintain constant water holding capacity. A sample of soil was taken from each microcosm after 1, 3, and 7 days following urea addition. Samples were freeze-dried within 3 h and stored at −80 °C for lipid extraction and DNA extraction. Phospholipid fatty acids (PLFAs) were extracted from soils 1 and 3 days after urea addition, and DNA were extracted from the soil samples taken after 7 days to analyze ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB).

2.4.1 Lipid extraction and PLFA analyses

Lipid extraction and PLFA analyses were performed using the modified Bligh and Dyer method (1993). Briefly, approximately 3.0000 g freeze-dried soil was extracted twice using 22.8 mL single-phase mixture (1:2:0.8 *v/v/v*) of chloroform: methanol and citrate buffer (0.15 M, pH 4.0). The phospholipids were separated from neutral and glycolipids on a silicic acid column and were methylated using a mild alkaline methanolysis to derivatize them to their respective fatty acid methyl esters (FAMES). The FAMES were separated on a gas chromatograph equipped with a flame ionization detector (MIDI Inc., Newark, DE, USA). Methyl nona decanoate fatty acid (19:0) was added, prior to the derivatization, as an internal concentration standard to quantify the phospholipids. The $^{13}C/^{12}C$ ratios of individual PLFAs were analyzed by GC-C-IRMS using a Trace GC Ultra gas chromatograph with combustion column attached via a GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (Thermo, Germany). The total ^{13}C enrichment rate (pt) of PLFAs in each soil were calculated according to the formulas described by Wang et al. (2014). PLFAs i15:0, a15:0, and i16:0 were used as biomarkers for Gram-positive bacteria (16:1ω7c, 18:1ω7c, and cy 19:0ω7c) and for Gram-negative bacteria (Wilkinson 1988) (18:2ω6c and 18:1ω9c) for saprophytic fungi, and 16:1ω5c for arbuscular mycorrhizal fungi (AMF) (Olsson 1999).

2.4.2 Quantification of AOA and AOB genes by real-time PCR

LightCycler®480 Real-Time PCR System (Roche Applied Science, CA) was used for real-time PCR. PCR primers used to amplify regions of functional genes from ammonia-oxidizing bacteria are Arch-amoAF, Arch-amoAR, amoA1F, and amoA2R. A typical 20 μL reaction contained 10 μL of SYBR® Premix Ex Taq™ II (TaKaRa), 0.4 μL each primer for final concentrations of each primer pair combination used. Details of the method have been given in Di et al. (2014).

2.5 Statistical analysis

Means and least significant difference at the 5 % significance level were calculated by one-way ANOVA. The molar percent of the individual PLFAs and percentage distribution of ^{13}C among total PLFA-C were standardized to unit variance (scaling) after generating a correction matrix and were then subjected to principal component analysis (PCA) to explain the variation of data. All statistical analyses were made using Excel 2010 and SPSS 20.0.

3 Results

3.1 The influence of different management practices on soil nutrient status

Basic chemical properties of soil samples are given in Table 1. There was substantial field variability of the soil properties among the replicate plots. Soil pH increased with soil depth, whereas soil available K, organic matter, and total N generally decreased with depth. In the 0–20 cm layer, the organic matter content in the E96 treatment was 52.3 % higher than in the FG treatment, and 36.0 % higher in the E83 than in the FG treatment. Total nitrogen in the E96 and E83 treatments was 53.2 and 55.7 % higher than in the FG treatment, at 0–20 cm, respectively, and was 17.3 and 26.9 % higher than in the FG treatment at 20–40 cm depth. The CEC of soil samples followed the order: E83>E96>FG at 0–20 cm. However, the management practices did not lead to consistent soil fertility changes in the subsoil layers.

3.2 Impact of management on microbial communities

The MBC followed the order E83>E96>FG in the 0–20 cm depth (Table 2). The MBN was similar in the E83 and E96 treatments and both were higher than in the FG treatment. The MBC in the 20–40 cm layer followed the order E96>E83>FG. There was no difference in MBN between the treatments at the 20–40 cm layer. However, at the 40–60 cm depths, the MBC and MBN were higher in the FG treatment than in the two enclosure treatments.

Table 1 Basic chemical properties of soil samples

Sample samples	pH	Available K mg kg^{-1}	Organic matter g kg^{-1}	Total nitrogen g kg^{-1}	CEC cmol kg^{-1}
	0–20 cm	0–20 cm	0–20 cm	0–20 cm	0–20 cm
E83	6.75±0.03a	141.50±1.88c	16.62±0.53b	1.33±0.12b	11.20±0.12a
E96	6.67±0.02b	178.10±1.29a	18.61±0.42a	1.53±0.08a	10.60±0.31b
FG	6.90±0.04a	164.38±1.15b	12.22±0.21c	1.18±0.05c	8.80±0.26c
	20–40 cm	20–40 cm	20–40 cm	20–40 cm	20–40 cm
E83	7.42±0.04b	44.53±0.81b	9.85±0.22b	0.76±0.14a	8.40±0.16b
E96	7.56±0.04b	58.68±0.90a	9.39±0.31b	0.71±0.09a	8.40±0.11b
FG	8.11±0.05a	58.57±1.45a	11.89±0.17a	0.64±0.21b	9.20±0.25a
	40–60 cm	40–60 cm	40–60 cm	40–60 cm	40–60 cm
E83	7.85±0.05b	32.57±0.70c	5.79±0.19c	0.44±0.07a	7.60±0.29b
E97	8.20±0.08a	41.80±0.99b	6.11±0.27b	0.47±0.11a	7.80±0.34b
FG	8.38±0.06a	61.39±1.41a	7.43±0.31a	0.49±0.15a	8.00±0.17a

Values followed by the same letter are not significantly different at $P \leq 0.05$ for the same depth. Data are presented as means±SE, $n=3$

3.3 Stable isotope probing

3.3.1 The diversity response of soil microbial community to urea addition

Soils under the E83 and FG treatments (only these two treatments were chosen) contained a variety of PLFAs comprising of saturated, unsaturated, methyl-branched, and cyclopropane fatty acids (Fig. 1). Twenty-four PLFAs with chain lengths from C14 to C20 were identified. The PLFA patterns varied in response to FG and E83 as revealed by their relative abundance (Fig. 1). Most ^{13}C in both the free-grazing and enclosed treatments was incorporated into i15:0, 16:1 ω7c, 16:1 ω5c, 16:0, 10-Me-16:0, and 18:1 ω7c; a lesser amount was also incorporated into a15:0, i16:0, i17:0, 18:1ω9c, and 18:0. The urea-derived C in 16:1ω5c from E83 was almost twice that from FG at the third day. These above lipids together accounted for 95 % of the total ^{13}C that was incorporated (Fig. 1c, d). Most ^{13}C incorporation into PLFAs at the third day decreased compared with day 1 in FG, and the ^{13}C incorporated into i16:0 in the E83 treatment declined sharply while increased in the FG treatment.

To investigate the effect of urea addition on soil microbial community composition of E83 and FG, PCA was performed on the molar percentage of individual PLFAs to the total PLFA pool (Fig. 2). The first principal component (PC1) accounted for 50.0 % of the variation and the second (PC2) showed a further 21.7 % variation as ^{12}C-PLFA (Fig. 2b). The PCA showed that the first principal component (PC1) accounted for 42.8 % of the variation and the second (PC2) accounted for 32.3 % variation as ^{13}C-PLFA (Fig. 2c). In contrast to percentage molar abundance of total (^{12}C+^{13}C) PLFAs, the

Table 2 Soil microbial biomass C and biomass N at three depths in three management practices

Sample samples	Microbial biomass C (mg kg^{-1})	Microbial biomass N (mg kg^{-1})
	0–20 cm	0–20 cm
E83	350.61±6.05a	29.39±0.03a
E96	304.20±3.51b	28.85±1.08a
FG	264.33±0.94c	19.73±0.01b
	20–40 cm	20–40 cm
E83	225.89±6.34b	23.35±0.12a
E96	249.76±1.97a	23.08±0.41a
FG	190.80±0.83c	22.45±0.22a
	40–60 cm	40–60 cm
E83	166.57±7.30b	20.03±0.79c
E96	162.24±3.33b	18.03±0.82b
FG	186.68±1.76a	27.04±0.75a

Values followed by the same letter are not significantly different at $P \leq 0.05$ for the same depth. Data are presented as means±SE, $n=3$

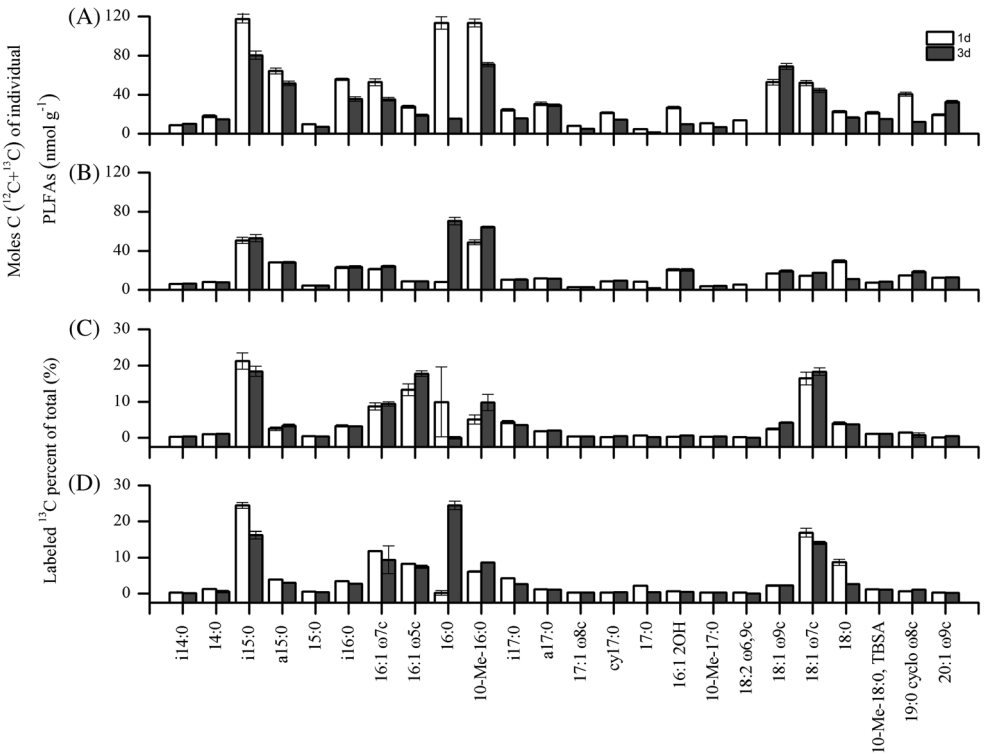

Fig. 1 Moles C of individual and labeled ^{13}C of the total PLFAs in E83 and FG. **a** Moles C in E83 after 1 and 3 days incubation, **b** moles C in FG after 1 and 3 days incubation, **c** relative abundance (mol %) of individual ^{13}C-PLFA from urea-derived ^{13}C in E83 after 1 and 3 days incubation, and **d** relative abundance (mol %) of individual ^{13}C-PLFA from urea-derived ^{13}C in FG after 1 and 3 days incubation

^{13}C incorporation showed no significant correlation between either PC1 or PC2 and soil management (Figs. 1a, b and 2c).

3.3.2 The growth response of ammonia oxidizers to urea addition

Ammonium-N concentration rapidly reached to a maximum due to urea hydrolysis and then decreased gradually. The nitrate-N concentration increased after the application of urea, following the nitrification of ammonium. The nitrate-N concentration in the FG treatment was much higher than that in the E83 in the third and seventh day (Fig. 3).

Following urea application, both AOB and AOA communities grew, as indicated by the increased abundance of *amoA* gene copy numbers after 3 and 7 days incubation (Fig. 4). However, the growth rate was higher in the soil from the FG treatment than in the soil from the E83 treatment. The growth rate is also higher for AOB than for AOA following urea application.

4 Discussion

4.1 Basic chemical properties

Results from this study clearly showed that pasture management had a major impact on soil properties and soil nutrient availability. Overall, soil nutrient fertility status increased when the grassland was enclosed; however, the fertility did not always continue to increase when the enclosure period increased from 1996 to 1983. This would indicate that as the enclosure period increased, the soil nutrient status would probably have reached a stable level and any further enclosure from grazing would probably not substantially change the soil nutrient status further (Ao et al. 2011). Therefore, there might be an optimum period of enclosure after which the grassland can be reverted back to grazing again. The challenge therefore is to determine the optimum enclosure that is required to restore the pasture and soil conditions. The decrease in soil pH in the surface layer of this alkaline soil was attributed to atmospheric acid deposition and plant root excretions in this alkaline soil (Grennfelt and Hultberg 1986). However, the impact of pasture management on the soil properties was mainly confined to the surface layer and the impact on subsurface soils was minimal.

4.2 Soil microbial diversity and biomass

Results from this study showed that pasture management also had an impact on microbial biomass. Microbial biomass was kept at a higher level under enclosure than under grazing, demonstrating that enclosure helped to restore the soil microbial biomass. Similar results have also been reported by other researchers (Holt 1997; Ma et al. 2005; Shrestha and Stahl 2008). For example, Holt (1997) reported that after 6–8 years of heavy beast grazing of two semi-arid grasslands in northeast Australia, soil microbial biomass decreased by about 24–51 %. Ma et al. (2005) also reported that after 22 years of

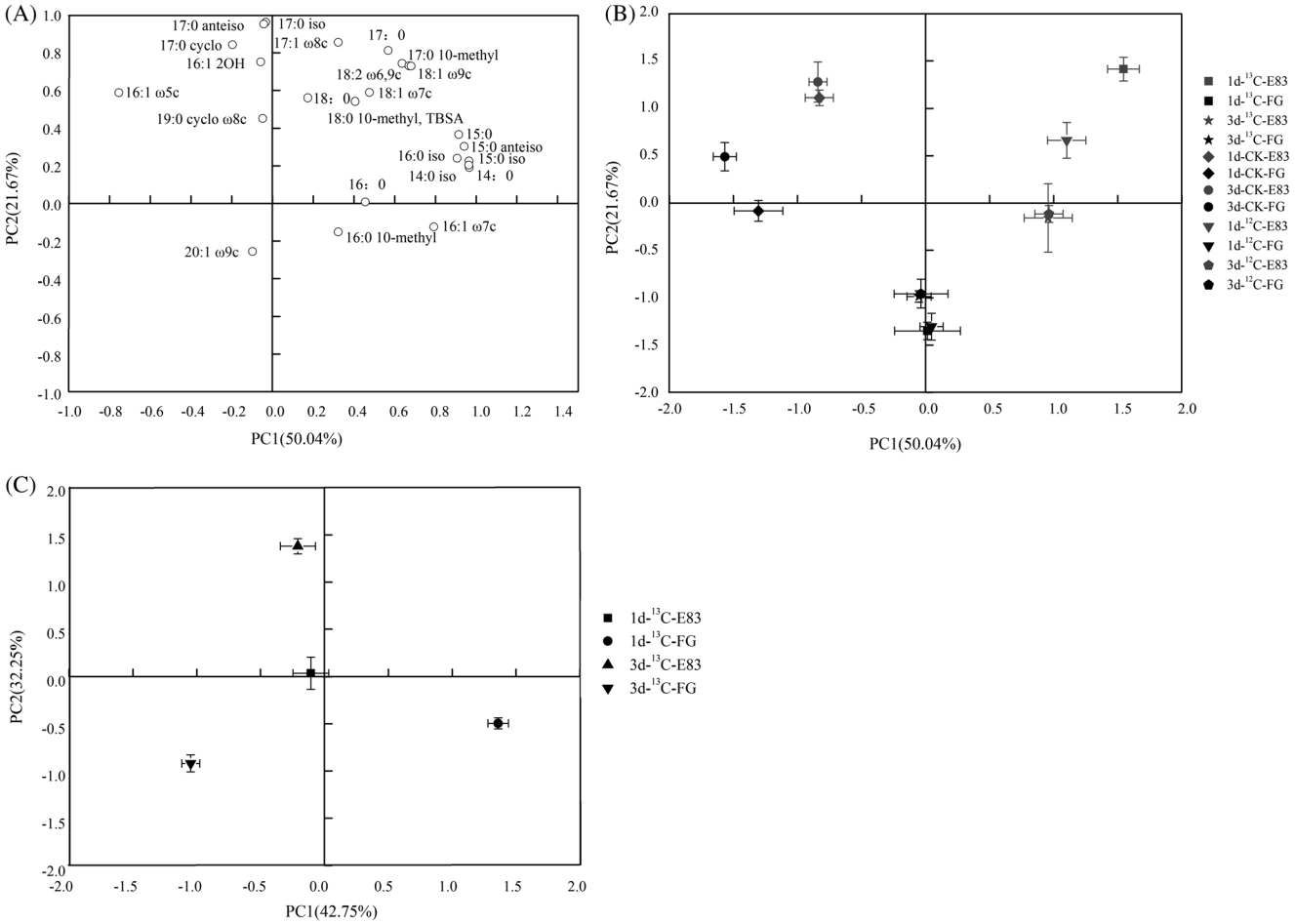

Fig. 2 **a** A loading plot of two components from the PCA on the PLFA composition. Percentage of variance explained by each component is indicated within parenthesis on each axis; **b** principal analysis of ^{12}C-PLFA composition at 1 and 3 days; **c** principal component analysis of ^{13}C-PLFA composition of soil samples at 1 and 3 das

sheep grazing of the *L. chinensis* grassland in Inner Mongolia, the surface soils' microbial biomass carbon decreased by about 27.9–38.2 %.

However, the species richness indices and diversity indices did not change significantly in the different treatments. Therefore, grazing decreased the microbial biomass, but did not result in major changes in diversity of the microbial communities.

4.3 Stable isotope probing microcosms

In the current study, the effect of urea on microbial community composition by ^{13}C-PLFA, through the addition of C and N, was much smaller than the effects of pasture management (Fig. 2), indicating that pasture management plays an important role in determining microbial composition of soils (Chaparro et al. 2012). However, the PCA charts of total PLFA molar abundance and ^{13}C-PLFA molar abundance (Figs. 1 and 2) indicated that urea addition did affect the allocation of urea-derived ^{13}C to microbes more than overall microbial community composition, and this agrees with findings reported by Hamer et al. (2009). Over 75 % of the urea-derived C was found in fungi 18:1ω9c, actinomycetes 10-Me-16:0, Gram-negative bacteria 16:1ω7c, 16:1ω5c, and Gram-positive bacteria i15:0, 16:0 (Fig. 1). PLFA 16:0 is widespread in different microbial populations, and is often used as a tool for the evaluation of total microbial biomass (Salomonová et al. 2003). The much higher incorporation of ^{13}C in PLFA 16:0 in the E83 soil after 3 and 7 days incubation, compared with the FG soil, would suggest higher metabolic activity in the E83 soil than the soil from the FG treatment.

It is interesting that the copy numbers of ammonia-oxidizing bacteria in the soil from the FG treatment was higher than that in the soil from the E83 treatment, indicating that ammonia-oxidizing bacteria communities might have been conditioned by the continued N cycling in the forms of urine and dung from the grazing animal. Therefore, the ammonia-oxidizing bacteria communities responded more rapidly following the addition of the N substrate in the form of urea. It is also interesting that both AOA and AOB grew following urea

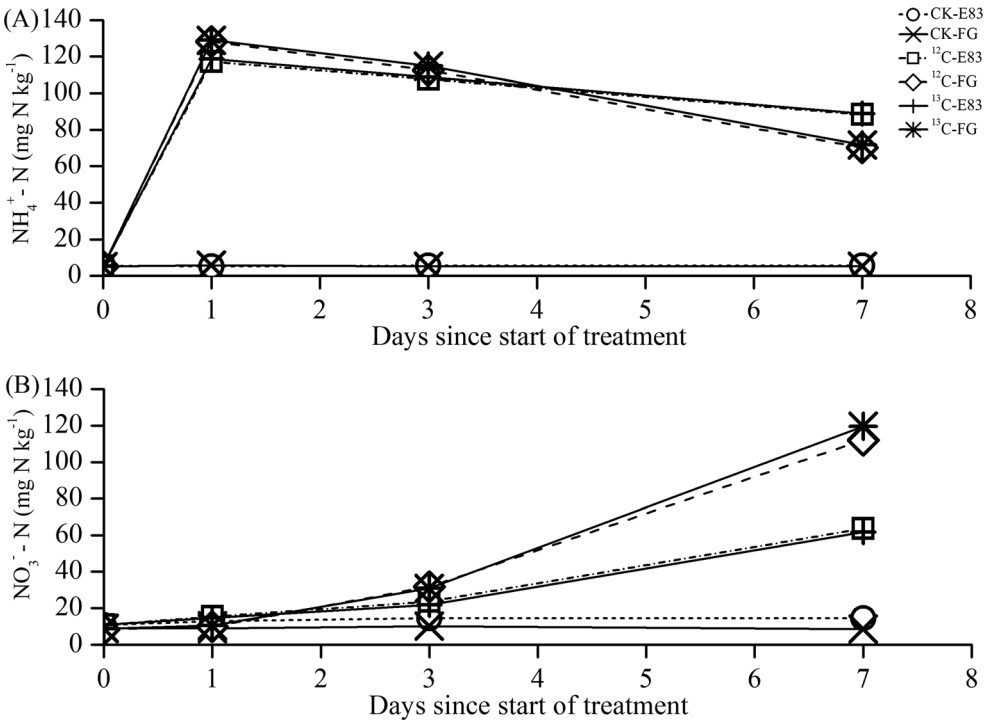

Fig. 3 Concentration of mineral N in the soil following treatment application: **a** ammonium N; **b** nitrate N

application. This was in contrast to earlier findings where AOB rather than AOA grew following the additions of animal urine on a fertile dairy pasture soil (Di et al. 2009, 2014). The AOB and AOA growth following urea application in this study may also reflect the low fertility nature of this grassland soil compared with the high fertility dairy pasture soils used in the study by Di et al. (2009, 2014). Therefore, in these semi-arid low fertility grassland soils, AOA may play a significant role in N cycling, and this aspect requires further investigation.

5 Conclusions

This study clearly showed that pasture management of semi-arid grasslands has a major impact on the soil fertility,

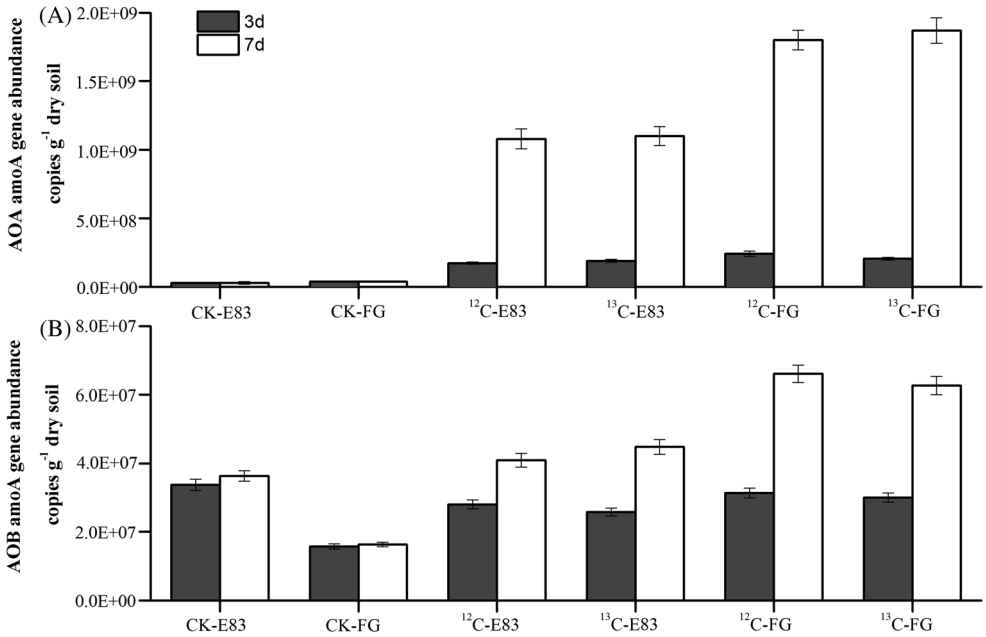

Fig. 4 Gene copy numbers after incubation. **a** archaeal amoA (*AOA*) and **b** bacterial amoA (*AOB*)

microbial biomass, and ammonia-oxidizing populations. Although soil nutrient status was higher in the soil under enclosure since 1997 but the soil nutrient status did not continue to increase with increased length of enclosure extending back to 1983. It is therefore important to define the optimum period for enclosure for different ecosystems. This study also showed that enclosure from animal grazing also had an impact on the microbial biomass but not on the microbial diversity. Enclosure also resulted in slower growth of ammonia-oxidizing bacteria in response to urine application. The bacteria i15:0, 16:1ω7c, 16:1ω5c, 16:0, 18:1ω7c, and actinomycetes 10-Me-16:0 used the most of ^{13}C-labeled urea. The implications of this on nutrient cycling and grassland ecosystem function requires further investigation.

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