Fungi regulate response of N_2O produce process to warming and grazing in a Tibetan grassland

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Abstract

Lack of understanding of the effects of warming and winter grazing on soil fungal contribution to nitrous oxide (N_2O) production process has limited our ability to predict N_2O fluxes under changes in climate and land use management, because soil fungi play an important role in driving terrestrial N cycling. A controlled warming and winter grazing experiment included control (C), winter grazing (G), warming (W) and warming with winter grazing (WG) was conducted to investigate the effects of warming and winter grazing on soil N_2O production potential in an alpine meadow on the Tibetan Plateau. Our results showed that soil bacteria and fungi contributed 46 ± 2 % and 54 ±2 % to nitrification, and 37 ± 3 % and 63 ± 3 % to denitrification in control treatment, respectively. We conclude that soil fungi could be the main source for N_2O production potential in the Tibetan alpine grasslands. In our results, neither warming nor winter grazing affected the activity of enzymes responsible for overall nitrification and denitrification. However, warming significantly increased the enzyme activity of bacterial nitrification and denitrification to 53% and 55%, respectively, but decreased enzyme activity of fungal nitrification and denitrification to 47% and 45%, respectively. Winter grazing had no such effects. Warming and winter grazing may not affect the soil N_2O production potential, but climate warming can alter biotic pathways responsible for N_2O production process. These findings confirm the importance of soil fungi in soil N_2O production process and how its responses to environmental and land use changes in alpine meadow ecosystems. Therefore, our results provide some new insights about

ecological controls on N_2O production process and contribute to the development of ecosystem nitrogen cycle model.

Keyword: warming, winter grazing, nitrification, denitrification, fungi

1 Introduction

N_2O emissions from soil contribute to climate warming as N_2O is a potent greenhouse gas (Change, 2015), it is mainly produced in soils through microbial nitrification and denitrification (Zumft, 1997). Clarifying nitrification and denitrification processes and their controlling factors will be beneficial for understanding N cycle in terrestrial ecosystems. Previous studies mainly focused on bacterial nitrification and denitrification (Hayatsu et al., 2008; Klotz and Stein, 2008) because the conventional N cycle is thought to be controlled primarily by bacteria. However, recent studies using novel molecular techniques have shown that soil fungi are important players in terrestrial N cycling, including N_2O production and nitrification/denitrification in drylands or soils with high organic carbon (C) and N (Chen et al., 2015; Huang et al., 2017; Laughlin and Stevens, 2002; Marusenko et al., 2013; Zhong et al., 2018).

The Tibetan grasslands occupy approximately 40% of the Tibetan Plateau which represents 0.7-1.0% of total global N storage (Tian et al., 2006) and is required for sufficient forage production (Zheng et al., 2000). These grasslands represent one of the most vulnerable regions in the world to climate change and anthropogenic perturbation (Thompson et al., 1993;Thompson, 2000;Wang and French, 1994). A much greater than average increase in the surface temperature has been predicted to occur in this region in the future (Giorgi et al., 2001) and have profound impacts on soil N cycling in alpine grasslands. Additionally, the grasslands of the Tibetan Plateau are generally divided into two grazing seasons, i.e. summer grazing from June to September and winter

grazing from October to May (Cui et al., 2014), which host about 13.3 million domestic yaks and 50 million sheep, with dramatically increasing numbers in future (Yao et al., 2006). Grazing strongly affects soil N cycling, as well as plant and microbial diversity (Hillebrand, 2008) and the stability of ecosystems (Klein et al., 2004). Previous studies have demonstrated losses of N caused by warming (Klein et al., 2004; 2007) and that overgrazing (Zhou et al., 2005) leads to degradation in alpine grasslands. The effects of climate warming and grazing on the aboveground vegetation, soil physicochemical properties, litter mass loss, bacterial communities and N_2O fluxes of Tibetan alpine grasslands have been extensively investigated (Hu et al., 2010;Li et al., 2016;Luo et al., 2010;Rui et al., 2012;Wang et al., 2012;Zhu et al., 2015); however, most of these studies was focused on the effect of summer grazing, little is showed the effect of winter grazing on them (Zhu et al. 2015; Che et al. 2018). On the other hand, many studies of Tibetan alpine grasslands are mainly focused on bacterial nitrifiers and denitrifiers or their activities, taking these to be the key factors on N_2O emission in alpine grasslands. However, while many studies have explored N mineralization, nitrification and even denitrification as well as bacterial nitrifiers and denitrifiers for better understanding of N_2O emission and ecosystem functioning (Yang et al., 2013;Yue et al., 2015), few studies have been conducted to distinguish whether bacteria or fungi dominate in N_2O emission and N cycling (Kato et al., 2013), especially under warming and grazing conditions.

Since optimum environments for fungi and bacteria are different, they may respond differently to environmental changes. Fungi prefer lower temperature (Pietikäinen et

al., 2005), higher organic C/N (Chen et al., 2015) and a more arid soil environment (Marusenko et al., 2013) compared to bacteria. Climate warming and grazing can change vegetation cover, soil water and energy balance, alter the quantity and quality of soil organic matter and mineral N content (Saggar et al., 2004), and thus affect N_2O production (Shi et al., 2017). However, it remains unknown how bacteria and fungi respond to concurrent warming and grazing and contribute to N_2O production in alpine grasslands.

To clarify whether fungi played the mainly role in N_2O produce process and its response to warming and winter grazing in alpine grasslands, we used a warming and grazing experiment over 10 years in an alpine meadow on the Tibetan Plateau. We measured the gene abundance of soil bacterial and fungal communities using quantitative PCR, and the potential of N_2O emission from bacterial and fungal nitrification and denitrification through an incubation experiment to assess the contribution of N_2O production potential from bacteria and fungi. We aimed to test the following hypotheses: (1) soil fungi were the main contributor to N_2O production because of the low soil temperature and high organic C and N in the alpine grasslands, and (2) although N_2O emission was not affected by warming and winter grazing at our site (Zhu et al., 2015), the biotic pathways responsible for N_2O would be changed due to the distinct preferred soil environments of bacterial and fungal communities.

2 Materials and Methods

2.1 Site and sampling. Details of the experimental site and design of the warming and

131 grazing were described by Wang et al. (2012). The experiment was conducted in an
132 alpine grassland (37°37'N, 101°12'E, 3250 m elevation) at the Haibei Alpine Meadow
133 Ecosystem Research Station of the Chinese Academy of Sciences. Over the past 25
134 years, the mean annual temperature was -2 ºC, and the mean annual precipitation was
135 500 mm. In the soil sampling year and month of 2015, mean temperature was 0 ºC and
136 9.7 ºC, respectively; total rainfall was 327.2 mm and 46.6 mm, respectively. Over 80%
137 of total rainfall falls during the summer monsoon season (Luo et al., 2010; Zhao and
138 Zhou, 1999). The soil was classified as Gelic Cambisols (WRB, 1998). The plant
139 community at the experimental site is dominated by *Kobresia humilis*, *Festuca ovina*,
140 *Elymus nutans*, *Poa pratensis*, *Carex scabrirostris*, *Gentiana straminea*, *Gentiana*
141 *farreri*, *Blysmus sinocompressus*, *Potentilla nivea and Dasiphora fruticosa* (Luo et al.,
142 2010).
143
144 A two-way factorial design (warming and grazing) was used with four replicates of
145 each of four treatments (Wang et al., 2012), beginning in May 2006, namely no
146 warming with no grazing (C), no warming with winter grazing (G), warming with no
147 winter grazing (W) and warming with winter grazing (WG). In total, 16 plots of 3-m
148 diameter were fully randomized throughout the study site.
149 For warming treatments, the design of the controlled warming (i.e. free-air
150 temperature enhancement (FATE) system with infrared heaters) with grazing
151 experiment described previously by Kimball et al.(2008) and Wang et al. (2012). Free-
152 air temperature enhancement using infrared heating has been set up to create a warming

153 treatment since May 2006 (Luo et al., 2010). The differences in canopy temperature at
154 set points between heated plots and the corresponding reference plots were 1.2ºC during
155 the daytime and 1.7ºC at night in summer. During winter, from October to April, the
156 power output of the heaters was manually set at 1500 W per plot, as some infrared
157 thermometers were not working.

158 For grazing treatments, during 2006-2010 summer grazing treatments was used to
159 explore the effects of warming and grazing on ecosystem during the growing seasons
160 (Luo et al. 2010; Hu et al. 2010; Wang et al. 2012). Considering strong disturbance,
161 grazing was stopped during 2011-2015, summer grazing was replaced by cutting and
162 removing about 50% of the litter biomass in October and the following March each
163 year to simulate winter grazing. Given the importance of winter grazing, winter grazing
164 during the non-growing seasons was further investigated (Zhu et al. 2015; Che et al.
165 2018). Alpine meadows in the region can be divided into two grazing seasons (i.e.,
166 warm-season grazing from June to September and cold-season grazing from October to
167 May) (Cui et al., 2015). Before the experiment was conducted, we had examined how
168 clipping simulated the effects of actual grazing before we established four replicated
169 “actual grazing treatments” compare with the “simulated grazing treatments”, the soil
170 and plant all showed no difference between simulated grazing and actual grazing
171 treatments (Klein et al. 2004; 2007), because the soil is frozen in winter, meaning that
172 the effect of selective feeding and trampling by sheep would be limited, so the effect of
173 cutting in winter was similar to winter grazing (Zhu et al., 2015).

174 **2.2 Soil sampling.** Five soil cores (5 cm in diameter) were randomly collected

within each plot on 15 August 2015 at a depth of 0–20cm (including organic layer) and then mixed to form a composite sample. All soil samples were transported to the laboratory and sieved through a 2-mm mesh before being stored at -20ºC or 4ºC for further molecular analyses.

2.3 Soil properties and gene abundance of bacteria and fungi analysis. Soil moisture content was measured by drying at 105°C for 24 hours. For soil mineral N (NH_4^+-N and NO_3^--N) analyses, 10 g of soil (field-moist) was shaken for 1 hour with 50 mL of 1 M KCl and filtered through filter paper, and determine the NH_4^+-N and NO_3^--N concentrations by Skalar flow analyzer (Skalar Analytical, Breda, The Netherlands). The total C and N content were measured by using combustion elemental analyzers (PerkinElmer, EA2400, USA).

Soil DNA was extracted from 0.5 g of the frozen soil using a FastDNA™ Kit for Soil (QBIOgene) based on the instructions and stored at -20°C. Total bacteria and fungi copies were quantified by real-time PCR using an iCycler thermal cycler equipped with an optical module (Bio-Rad, USA)

The real-time PCR mixture contained 5 ng of soil DNA, 2 pmol of primers and 10×iQ SYBR Green super mix (Bio-Rad), in a 20-μL reaction volume. The primer for bacteria were 341F 5'-CCTACGGGAGGCAGCAG-3' and 534R 5'-ATTACCGCGGCTGCTGGCA-3' (Muyzer et al., 1993). The thermal cycle conditions were 10 min at 95°C; 35 cycles of PCR were then performed in the iCycler iQ Real-

196 Time PCR Detection System (BIORAD) as follows: 20 s at 95°C, 15 s at 55°C and 30
197 s at 72°C. A final 5-min extension step completed the protocol. The primer for fungi
198 were FU18S1 5'-GGAAACTCACCAGGTCCAGA-3' derived from Nu-SSU-1196
199 and Nu-SSU-1536 5'-ATTGCAATGCYCTATCCCCA-3' (Borneman and Hartin, 2000)
200 and the thermal cycle conditions were one step of 10 min at 95°C, then 40 cycles of
201 PCR performed as follows: 20 s at 95 ºC, 30 s at 62 ºC and 30 s at 72 ºC. A final 5-min
202 extension step completed the protocol.

203 **2.4 Total, fungal and bacterial nitrification enzyme activity, and total, fungal and**
204 **bacterial potential of N_2O production from denitrification analysis.**

205 Fungal (FNEA), bacterial (BNEA) and total nitrification enzyme activity (TNEA)
206 was determined following the protocol described in Dassonville et al. (2011). Briefly,
207 moist field soil equivalent to 12 g of dry soil was weighed into 240-mL specimen bottles
208 (LabServ), 12 mL of NH_4-N solution (50 µg N-$(NH_4)_2SO_4$ g^{-1} dry soil) and distilled
209 water was added to achieve a 96 mL total liquid volume, and the slurry was incubated
210 at 28°C for 10 hours with constant agitation (180 rpm) in an orbital shaker (Lab-Line
211 3527; Boston, MA, USA) to mix slurry well and provide an aerobic environment. Three
212 treatments were imposed: (I) cycloheximide ($C_{15}H_{23}NO_4$, a fungicide) at 1.5 mg g^{-1} in
213 solution was used to inhibit the nitrification activity from soil fungi, (II) streptomycin
214 sulphate ($C_{42}H_{84}N_{14}O_{36}S_3$, a bactericide) at 3.0 mg g^{-1} in solution was used to inhibit
215 the nitrification activity from soil bacteria (Castaldi and Smith, 1998;Laughlin et al.,
216 2009) and (III) a no-inhibitor control was used to show the total nitrification activity.

During incubation, 10 mL of the soil slurry was sampled with a syringe at 2, 4, 6, 8 and 10 h, and then filtered through Whatman No. 42 ashless filter paper. Filtered samples were stored at -20 °C until analysis for NO_2^-+NO_3^- concentration on a LACHAT Quickchem Automated Ion Analyzer (Foss 5027 Sampler, TECATOR, Hillerød, Denmark). A linear regression between the NO_2^-+NO_3^- production rate and time was observed, and the rates of nitrification enzyme activity were determined from the slope of this linear regression. The nitrification enzyme activity of soil fungi was estimated by the difference between rates of nitrification enzyme activity under treatment (III) and treatment (I); the nitrification enzyme activity of soil bacteria was estimated by the difference between rates of nitrification enzyme activity under treatment (III) and treatment (II). The total nitrification enzyme activity was from treatment III.

Fungal (FDEA), bacterial (BDEA) and total potential of N_2O production (TDEA) from denitrification was measured in fresh soil from each plot following the protocol described in Patra et al. (2006) and Marusenko et al. (2013). Three sub-samples (equivalent to 12 g dry soil) from each soil sample were placed into 240-mL plasma flasks, and 7 mL of a solution containing KNO_3 (50 μg NO_3^-N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) were added. Additional distilled water was provided to achieve 100% water-holding capacity and optimal conditions for denitrification. Three treatments were imposed: (I) cycloheximide ($C_{15}H_{23}NO_4$; a fungicide) at 1.5 mg g^{-1} in solution was used to inhibit the fungal potential of N_2O production from denitrification, (II) streptomycin sulphate ($C_{42}H_{84}N_{14}O_{36}S_3$; a bactericide) at 3.0 mg g^{-1} in solution was used to inhibit the bacterial

potential of N_2O production from denitrification (Castaldi and Smith, 1998;Laughlin and Stevens, 2002), and (III) a no-inhibitor control was used to show the total potential of N_2O production from denitrification. The headspace air of the specimen bottles was replaced with N_2 to provide anaerobic conditions. Specimen bottles were then sealed with a lid containing a rubber septum for gas sample collection. Specimen bottles with the soil slurry were then incubated at 28°C for 48 h with constant agitation (180 rpm) in an orbital shaker (Lab-Line 3527; Boston, MA, USA). During incubation, 12-mL gas samples were taken at 0, 24 and 48 h with syringes and injected into pre-evacuated 6-mL glass vials. The N_2O concentration of the gas samples was analyzed via gas chromatography. The potential of N_2O production from denitrification were calculated from the slope of the regression using values for 0, 24 and 48 hours of incubation. The fungal potential of N_2O production from denitrification was estimated by the difference between rates of denitrification enzyme activity under treatment (III) and treatment (I); The bacterial potential of N_2O production from denitrification was estimated by the difference between rates of denitrification enzyme activity under treatment (III) and treatment (II). Total denitrification enzyme activity was from Treatment III.

For the contribution of bacteria and fungi to total nitrification enzyme activity was calculated it by the ratio of BNEA or FNEA to BNEA+FNEA; the contribution of bacteria and fungi to total potential of N_2O production from denitrification was calculated it by the ratio of BDEA or FDEA to BDEA+FDEA.

2.5 Statistical analysis. For the controlled experiment, the statistical significance of

the effects of warming, grazing and their interaction on plant biomass, soil properties, microbial functional genes, and fungal and bacterial nitrification enzyme activity and potential of N_2O production from denitrification were tested by two-way ANOVA in the PROC GLM procedure of SAS (version 9, SAS Institute, Cary, NC, USA).

3 Results

3.1 Plant biomass and soil properties

The average plant standing biomass was 343, 345, 301 and 362 g dry matter m^{-2} in the control, G, W and WG treatments measured at the day of soil sampling, respectively. Grazing and warming had no effect on plant biomass (Fig. 1a, Table 1).

Soil temperature varied from 11.8 to 14.0 ^{o}C. Grazing ($P=0.05$) and warming ($P<0.01$) increased soil temperature (Fig. 1b, Table 1). The average soil moisture varied from 26% to 34% (w/w). Grazing had no effect on soil moisture, which was lower in warming plots ($P<0.01$) (Fig. 1c, Table 1). There was an interactive effect between grazing and warming on soil temperature ($P<0.01$).

Soil total C (TC) was not affected by grazing ($P=0.13$) or warming ($P=0.12$) alone, but there was a marginal interaction between grazing and warming on TC ($P=0.07$) (Fig. 2a, Table 1). Similar to TC, soil total N (TN) also showed no response to grazing or warming (Fig. 2b, Table 1). Soil NH_4^+-N content was lower in warming treatments than in no-warming treatments ($P=0.05$) (Fig. 2c, Table 1). Greater soil NO_3-N content occurred under the warming treatments ($P=0.05$) than under the no-warming treatments (Fig. 2d, Table 1).

3.2 Microbial functional genes

Bacterial gene abundance varied from 4.71×10^9 to 5.93×10^9 copies g^{-1} dry soil, which was much higher than fungal gene abundance (Fig. 3). Warming and grazing both increased the bacterial gene abundance in soils ($P<0.01$), but there was no interaction effect between them on bacterial gene abundance (Table 1). By comparison, fungal gene abundance showed no difference across all treatments.

3.3 Nitrification enzyme activity and potential of N_2O production from denitrification of bacteria and fungi

TNEA varied from 1.07 to 1.64 μg N g^{-1} h^{-1} in all treatments. BNEA ranged from 0.43 to 0.64 μg g^{-1} h^{-1}, which was lower than the FNEA in soils (0.59–0.66 μg g^{-1} h^{-1}) ($P=0.01$) (Fig.4 a-c). FNEA was lower under warming treatments than under the no-warming treatments ($P=0.05$) (Table 1).

TDEA was between 1.32 and 1.80 μg N g^{-1} h^{-1}. FDEA was clearly the dominant process for TDEA (Fig. 4 d-f), because it was higher than BDEA for all treatments except warming. Warming increased BDEA ($P=0.04$). Warming and grazing had a significant interaction effect on FDEA ($P<0.01$) (Table 1).

3.4 The contribution of bacteria and fungi to potential N_2O emissions

The contribution of FNEA to TNEA varied from 47% to 56%, and the contribution of FDEA to TDEA varied from 45% to 63% (Fig. 5). Warming significantly decreased

302 the contribution of FNEA and FDEA to TNEA and TDEA in soils (FNEA: P=0.02;
303 FDEA: P=0.04). There were no differences in the contribution of FNEA and FDEA to
304 TNEA and TDEA in any treatments (Table 1).
305
306 **4 Discussion**
307 N_2O is mainly produced from the microbial nitrification and denitrification processes,
308 but the contribution of bacteria and fungi to nitrification and denitrification processes
309 is still unclear. In our results, fungi contributed 54% and 63% of the TNEA and TDEA,
310 respectively, in control treatment of the alpine grassland studied. Our result of the
311 fungal contribution to potential of N_2O production is much lower than Laughlin and
312 Stevens (2002) and Zhong *et al.* (2018) whom reported 89% and 86% fungal
313 contribution from temperate grasslands, but is higher than the 40-51% fungal
314 contribution observed across different ecosystems by Chen et al. (2014). Kato et al.
315 (2013) also showed that N_2O emissions from FDEA was higher than from BDEA in
316 alpine meadows, reinforcing the important role fungi play in denitrification process.
317 Our findings support our first hypothesis and further proved that both nitrification and
318 denitrification were largely driven by fungal communities in alpine meadow grasslands.
319 A possible explanation is that fungi prefer the arid, high organic substrate and low-
320 temperature environment (Pietikäinen et al., 2005;Chen et al., 2015;Marusenko et al.,
321 2013). In alpine grasslands, the mean annual temperature is 0 ^{o}C; even during the
322 sampling day the mean temperature was only 11 ^{o}C. The cold environment could cause
323 higher activity in fungi than in bacteria. Moreover, the cold environment decreases the
324 rate of mineralization, leading to greater organic C and N accumulation (Ineson et al.,

1998;Schmidt et al., 2004). In our study, soil TC and TN concentrations were 72–86 g kg^{-1} and 6–7 g kg^{-1}, respectively (Fig. 2a and 2b), much higher than in temperate grasslands and farmland, providing a favorable environment for fungi (Bai et al., 2010). These are mainly reasons that soil fungi played the mainly role in N_2O production process in the Tibetan alpine grasslands.

Our methodology did not exclude a role for archaea in nitrification and denitrification. Previous studies on grasslands only focused on fungal and bacterial process because archaeal specific inhibitors have not yet been identified for N cycling processes. However, archaea are widespread in soils, are involved in nitrification denitrification (Cabello et al., 2004), eg. archaeal ammonia oxidizers are globally (Leininger et al., 2006). In our study, we also found the TNEA was higher than the sum of NEA from bacteria and fungi, while TDNA was higher than DEA from bacteria and fungi (Fig. 4), which showed that archaea also played the role in N_2O production process in our site. However, it included the archaeal and abiotic components. The development of inhibitor-based approaches may help to show how archaea responses to environmental change (Marusenko et al. 2013).

Our results supported the second hypothesis that although warming did not change the total N_2O production potential on the Qinghai-Tibetan Plateau, the biotic pathways responsible for N_2O had been changed, as bacterial contribution to TNEA and TDEA all were higher than fungal which suggested the higher bacterial N_2O production potential under warming treatment (Fig. 4, Table 1). The increase in bacterial N_2O production potential, coupled with decrease in fungal N_2O production potential, could

347 be the main reason why the total N_2O production potential was no difference between
348 control and warming treatments. The field data in our site was measured in year of
349 2011–2012 and also showed no effect of warming on N_2O emission (Zhu et al. 2015).
350 Our results reinforced this and suggested that bacterial nitrification and denitrification
351 process alone is unable to accurately describe the response of N_2O to warming.
352 It is the two reasons that lead to the changes of fungal and bacterial pathways for
353 N_2O production process by warming. Firstly, warming significantly increased the soil
354 temperature (Fig.1b, Table 1), the increased of soil temperature directly reduce fungal
355 activity but increase bacterial activity, because fungi prefer the cold environment
356 compared with bacteria (Pietikäinen et al., 2005). Secondly, fungi prefer higher organic
357 C/N environment while bacteria prefer higher inorganic C/N environment (Chen et al.,
358 2015). In our site, although the soil NH_4^+-N concentration did not change with warming,
359 soil NO_3^--N concentration was significantly increased showed the soil inorganic N was
360 increased (Fig. 2a and 2b, Table 1); on the other hand, the soil dissolved organic
361 nitrogen was significantly decreased from 48 to 41 mg kg^{-1} ($P<0.04$), the soil labile C
362 and N was also found significantly decreased by warming (Rui et al., 2012), it showed
363 the soil organic C and N was decreased in our site. Therefore, warming indirectly reduce
364 fungal activity but increase bacterial activity through increased soil inorganic N and
365 decreased soil organic N in our site. In our site, the FNEA and FDEA were reduced by
366 16% and 30% respectively, but the BNEA and BDEA was increased by 15% and 41%
367 respectively by warming. All these changes resulted in fungi contributing less to
368 nitrification and denitrification than bacteria (Fig.5). Although the gene abundance of

fungi was not changed by warming which showed inconsistencies with the changes of FNEA and FDEA, these inconsistencies might be explained by the fungal gene abundance was not likely provided information on real-time process rates since such rates are dependent on environmental conditions, fluctuations in environmental conditions can cause rapid changes in real-time process rates, but not necessarily affect gene abundance (Zhong et al. 2014). In summary, it indicates that the soil microbial process was altered by warming, even though the total potential of N_2O production did not change, with a shift in the dominance from fungi to bacteria in N_2O production process after 10 years of warming.

Numerous studies have demonstrated that grazing can impact microbial processes and induce the loss of N through: (1) altering the substrate concentration for N_2O production and reduction in soil through the deposition of dung and urine (Saggar et al., 2004); (2) reducing vegetation cover due to changes in soil water content and energy balance (Leriche et al., 2001); and (3) increasing soil compaction and reducing soil aeration through animal tramping (Houlbrooke et al., 2008). However, most of these were focused on grazing in growing seasons, little was focused on the effect of winter grazing on N cycle process. In this study fungal and bacterial potential of N_2O production from nitrification and denitrification all showed little response to winter grazing (Fig. 4, Table 1). A possible explanation is that neither soil moisture, plant biomass nor organic/inorganic C/N content were affected by winter grazing (Fig.1-2, Table 1). Additionally, the soil was frozen in winter, so that the effect of selective feeding and trampling could be limited by grazing sheep rather than other livestock

391 (Zhu et al., 2015; Krümmelbein et al., 2009; Steffens et al., 2008). As a result, the same
392 soil environmental conditions for both winter grazing and control had no effect on soil
393 fungi and bacteria, and thus on fungal and bacterial nitrification and denitrification.
394 Moreover, the field data of N_2O emission in the year of 2011-2012 also supports the
395 results of Zhu et al. (2015) and suggests that replacing summer grazing by winter
396 grazing could cause the soil N cycle process to become stable.

397 Overall, we conclude that fungi played the dominant role in the N_2O production
398 process in alpine meadows. Previous study had proved the climate warming did not
399 affect the N_2O production in our site (Zhu et al. 2015), but we found warming could
400 alter biotic pathways responsible for N_2O production process on the Tibetan Plateau.
401 Our study exhibited the effects of a decade of simulation experiment; however, a
402 thorough understanding about the long-term impact of warming and grazing on soil
403 fungal nitrification and denitrification from alpine meadow grassland requires further
404 investigation for multi-decade period.

405 From this study, due to the different adaptation strategies of fungi and bacteria, and
406 their different nutrition requirements, future changes in climate and soil resources are
407 likely to affect biogeochemistry in a way not currently accounted for in ecosystem
408 models that assume N transformations are controlled only by bacteria. Accurate
409 predictions for N_2O production and N loss due to environmental change and land use
410 will benefit from the inclusion of fungi as key mediators of ecological processes in
411 grasslands.

412

Competing interests

The authors declare that they have no conflict of interest.

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Table 1. Results (F-value and P-value) from two-way ANOVA for the effects of warming (W), winter grazing (G) and their interactions (WG) on soil and microbial characteristics.

	W		G		WG	
	F value	P value	F value	P value	F value	P value
Biomass	0.21	0.65	1.41	0.26	1.21	0.29
Temperature	61.16	**<0.01**	4.64	**0.05**	25.54	**<0.01**
Soil moisture	14.87	**<0.01**	0.17	0.68	0.13	0.72
TC	2.69	0.12	2.7	0.13	3.95	0.07
TN	1.44	0.25	1.47	0.25	3.02	0.11
NH_4^+-N	4.57	**0.05**	1.6	0.23	0.02	0.89
NO_3^--N	3.6	0.05	1.42	0.25	0.09	0.81
Bacterial	17.91	**<0.01**	11.67	**<0.01**	0.11	0.75
Fungi	1.72	0.21	0.70	0.42	2.89	0.12
BNEA	1.01	0.90	3.24	0.35	3.94	0.07
FNEA	4.58	**0.05**	1.15	0.34	0.37	0.51
TNEA	0.8	0.39	2.23	0.16	0	0.95
BDEA	5.16	**0.04**	2.45	0.14	4.04	0.07
FDEA	1.52	0.24	0.96	0.34	9.98	**<0.01**
TDEA	0.98	0.34	2.33	0.15	0.15	0.70

Bold indicates significance at $P < 0.05$.

Figure caption

Fig. 1, Plant biomass (a) soil temperature (b) and soil moisture content (c) in an alpine meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).

Fig. 2 Soil total carbon (TC) (a), soil total nitrogen (TN) (b), soil NH_4^+-N (c) and NO_3^--N (d) content in an alpine meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).

Fig. 3 Abundance of bacteria (a) and fungi (b) in an alpine meadow; C (■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).

Fig. 4 Bacterial nitrification enzyme activity (BNEA) (a), fungal nitrification enzyme activity (FNEA) (b), total nitrification enzyme activity (TNEA) (c); Bacterial potential of N_2O production from denitrification (BDEA) (d), fungal potential of N_2O production from denitrification (FDEA) (e) and total potential of N_2O production from denitrification (TDEA) (f) in an alpine meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).

Fig. 5 Contribution of bacteria and fungi to total nitrification enzyme activity (box with the red and dashed line) and total potential of N_2O production from denitrification (box with the black and solid line) in an alpine meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).

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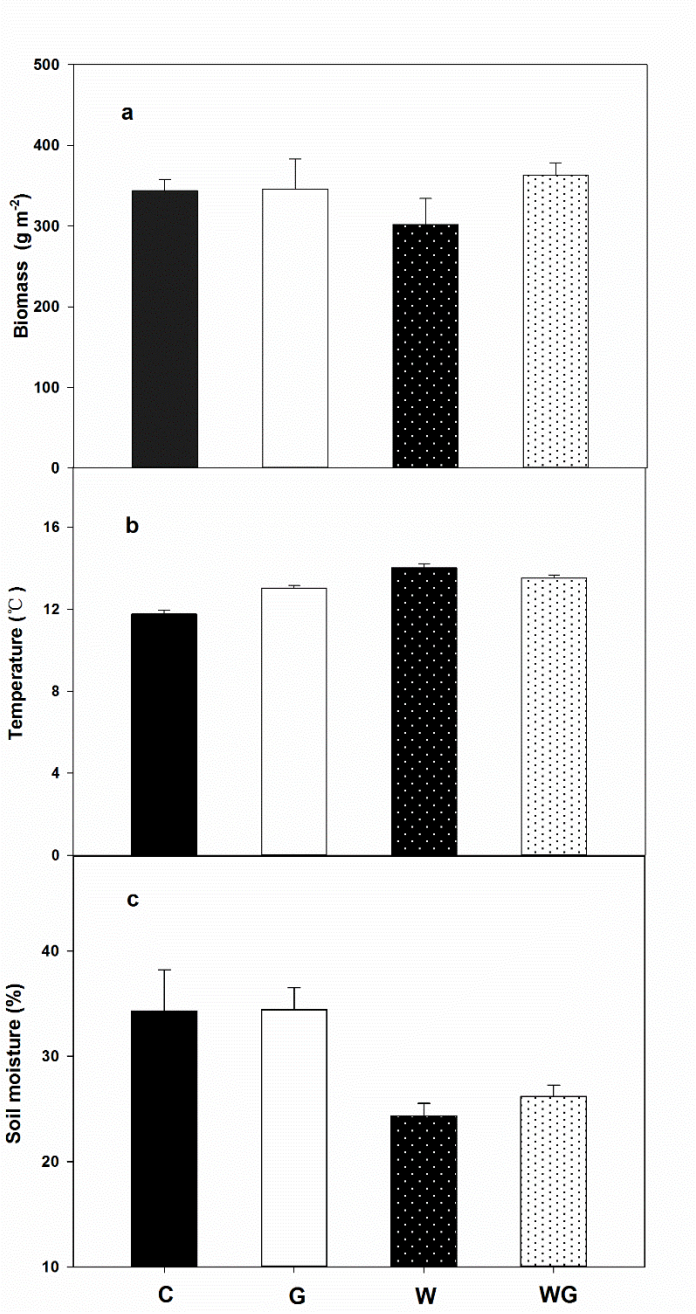
622 Fig.1



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625 Fig. 2

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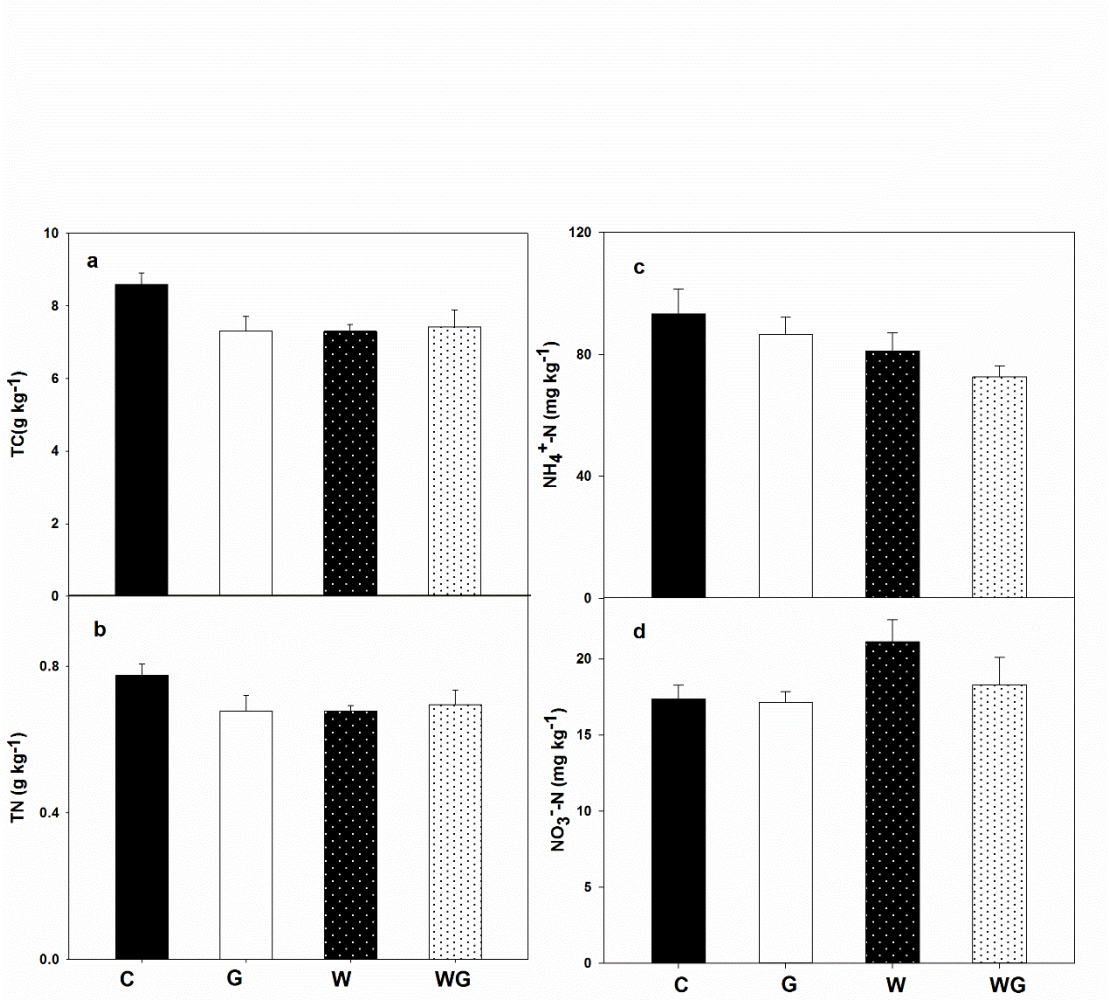

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630 Fig. 3
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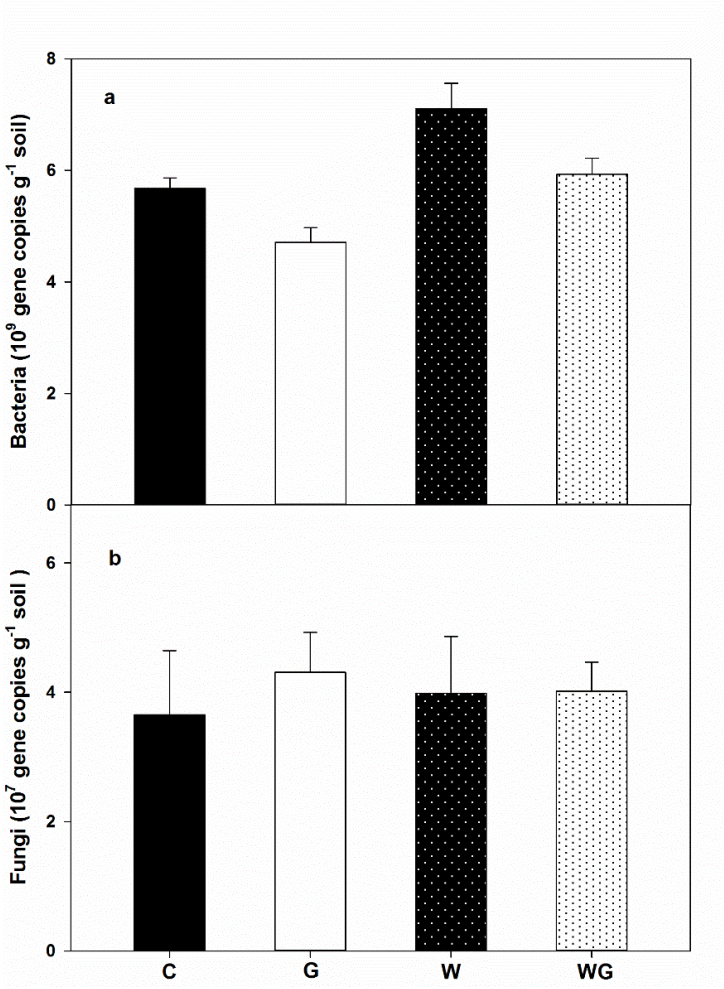

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637 Fig.4

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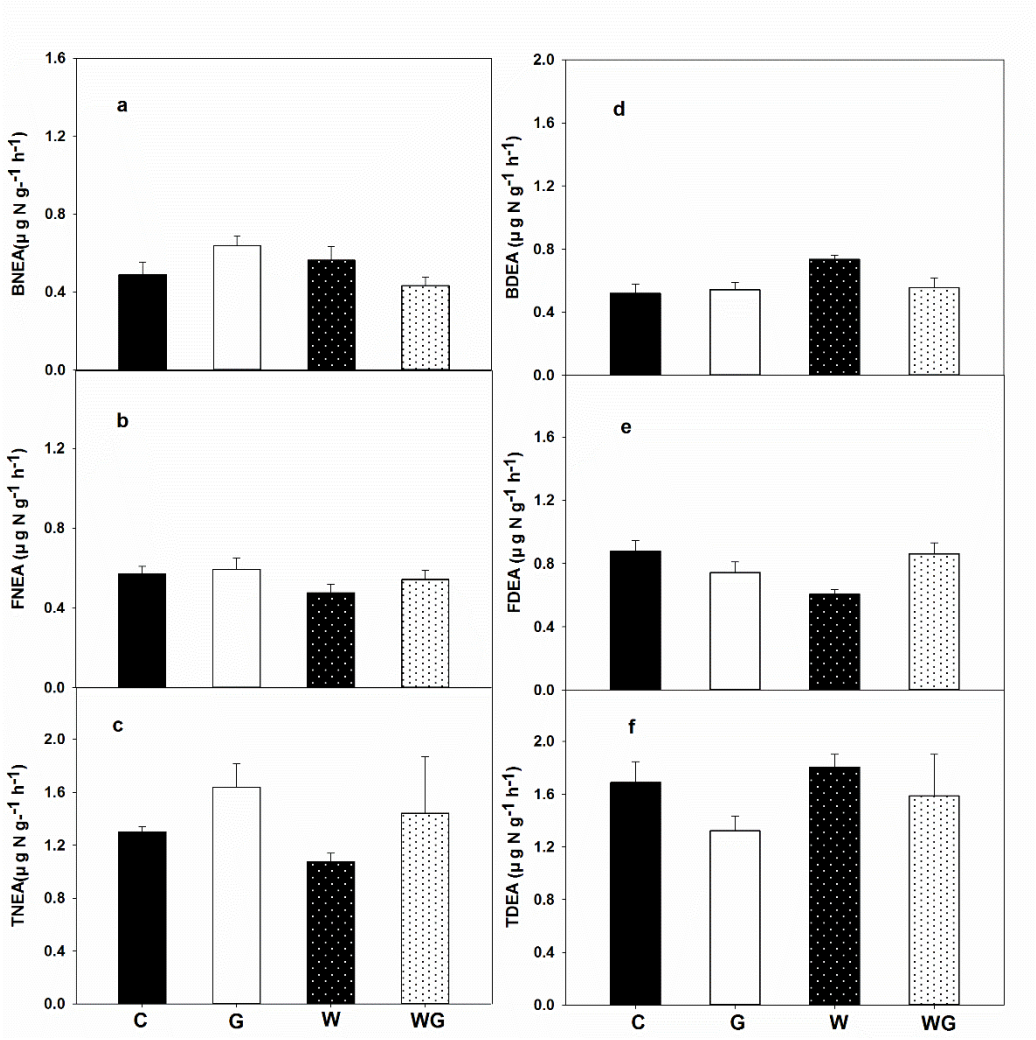

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641 Fig.5

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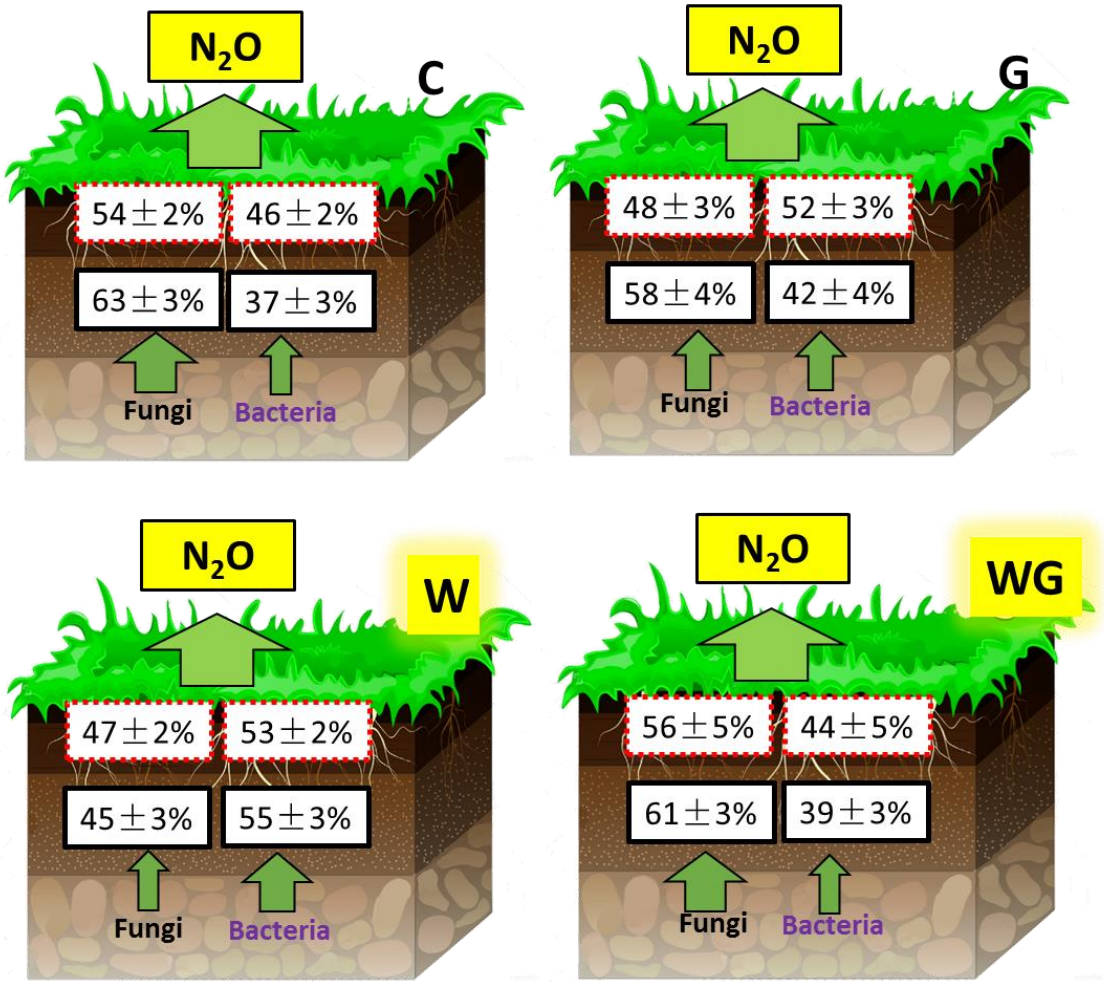

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