Fungi regulate response of N_2O production 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 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. Here, we examined the effects of 10 years' warming and winter grazing on soil N_2O emissions potential in an alpine meadow. Our results showed that soil bacteria and fungi contributed 46% and 54 % to nitrification, and 37% and 63% to denitrification, respectively. 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. Warming significantly decreased enzyme activity of fungal nitrification and denitrification to 47% and 45%, respectively, while winter grazing had no such effect. We conclude that soil fungi could be the main source for N_2O production potential in the Tibetan alpine grasslands. Warming and winter grazing may not affect the potential for soil N_2O production potential, but climate warming can alter biotic pathways responsible for N_2O production. These findings indicate that characterizing how fungal nitrification/denitrification contributes to N_2O production, as well as how it responds to environmental and land use changes, can advance our understanding of N cycling. Therefore, our results provide some new insights about ecological controls on N_2O production and lead to refine greenhouse gas flux models.

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

1 **Introduction**

Nitrogen losses through 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 the loss of N and climate warming via N_2O and its controlling factors will be beneficial for understanding N limitation and climate warming occurring 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 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) . Many studies of Tibetan alpine grasslands are mainly focused on bacteria 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 control the N_2O production 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 grazing were described by Wang et al. (2012). The experiment was conducted in an alpine grassland (37°37'N, 101°12'E, 3250 m elevation) at the Haibei Alpine Meadow Ecosystem Research Station of the Chinese Academy of Sciences. Over the past 25 years, the mean annual temperature was -2^{o}C, and the mean annual precipitation was

500 mm. In the soil sampling year and month of 2015, mean temperature was 0 ℃ and 9.7 ℃, respectively; total rainfall was 327.2 mm and 46.6 mm, respectively. Over 80% of which falls during the summer monsoon season (Luo et al., 2010; Zhao and Zhou, 1999). The soil type belongs to Mat-Gryic Cambisol, corresponding to Gelic Cambisol(Cao et al., 2008). The plant community at the experimental site is dominated by *Kobresia humilis*, *Festuca ovina*, *Elymus nutans*, *Poa pratensis*, *Carex scabrirostris*, *Gentiana straminea*, *Gentiana farreri*, *Blysmus sinocompressus*, *Potentilla nivea and Dasiphora fruticosa* (Luo et al., 2010).

A two-way factorial design (warming and grazing) was used with four replicates of each of four treatments (Wang et al., 2012), beginning in May 2006, namely no warming with no grazing (C), no warming with winter grazing (G), warming with no winter grazing (W) and warming with winter grazing (WG). In total, 16 plots of 3-m diameter were fully randomized throughout the study site.

For warming treatments, the design of the controlled warming (i.e. free-air temperature enhancement (FATE) system with infrared heaters) with grazing experiment was described previously by Kimball et al.(2008) and Wang et al. (2012). Free-air temperature enhancement using infrared heating has been set up to create a warming treatment since May 2006 (Luo et al., 2010). The differences in canopy temperature at set points between heated plots and the corresponding reference plots were 1.2^{o}C during the daytime and 1.7^{o}C at night in summer. During winter, from October to April, the power output of the heaters was manually set at 1500 W per plot,

as some infrared thermometers were not working.

For grazing treatments, the grazing treatments in this site were used for summer grazing treatments until 2010, from 2011 to 2015, there was no grazing during the summer, and grazing was replaced by cutting and removing about 50% of the litter biomass in October and the following March each year to simulate winter grazing. In our field, the soil is frozen in winter, meaning that the effect of selective feeding and trampling by sheep would be limited, so the effect of cutting in winter was similar to winter grazing (Zhu et al., 2015). Alpine meadows in the region can be divided into two grazing seasons (i.e., warm-season grazing from June to September and cold-season grazing from October to May) (Cui et al., 2015). In our field, the experimental platform showed the effects of warming and winter grazing on ecosystems in an alpine meadow grassland.

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 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 -20^{o}C or 4^{o}C 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-Time PCR Detection System (BIORAD) as follows: 20 s at 95°C, 15 s at 55°C and 30 s at 72°C. A final 5-min extension step completed the protocol. The primer for fungi were FU18S1 5'-GGAAACTCACCAGGTCCAGA-3' derived from Nu-SSU-1196 and Nu-SSU-1536 5'-ATTGCAATGCYCTATCCCCA-3' (Borneman and Hartin, 2000) and the thermal cycle conditions were one step of 10 min at 95°C, then 40 cycles of PCR performed as follows: 20 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C. A final 5-min extension step completed the protocol.

2.4 Potential total nitrification/denitrification enzyme activity, and fungal and bacterial nitrification/denitrification enzyme activity analysis.

Fungal, bacterial, and total nitrification enzyme activity was determined following the protocol described in Dassonville et al. (2011). Briefly, moist field soil equivalent to 12 g of dry soil was weighed into 240-mL specimen bottles (LabServ), 12 mL of NH_4-N solution (50 µg N-$(NH_4)_2SO_4$ g^{-1} dry soil) and distilled water was added to achieve a 96 mL total liquid volume, and the slurry was incubated at 28°C for 10 hours with constant agitation (180 rpm) in an orbital shaker (Lab-Line 3527; Boston, MA, USA) to mix them well and provide an aerobic environment. 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 nitrification activity from soil fungi, (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 nitrification activity from soil bacteria (Castaldi and Smith, 1998;Laughlin et al., 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, bacterial, and total nitrification enzyme activity 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 denitrification activity from soil fungi, (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 denitrification activity from soil bacteria, (Castaldi and Smith, 1998;Laughlin and Stevens, 2002) and (III) a no-inhibitor control was used to show the total denitrification activity. 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 rates of

239 denitrification enzyme activity were calculated from the slope of the regression using
240 values for 0, 24 and 48 hours of incubation. The denitrification enzyme activity of soil
241 fungi was estimated by the difference between rates of denitrification enzyme activity
242 under treatment (III) and treatment (I); the denitrification enzyme activity of soil
243 bacteria was estimated by the difference between rates of denitrification enzyme
244 activity under treatment (III) and treatment (II). Total denitrification enzyme activity
245 was from Treatment III. For the contribution of bacteria and fungi to total nitrification
246 enzyme activity was calculated it by the ratio of BNEA or FNEA to BNEA+FNEA; the
247 contribution of bacteria and fungi to total denitrification enzyme activity was calculated
248 it by the ratio of BDEA or FDEA to BDEA+FDEA.

249 **2.5 Statistical analysis.** For the controlled experiment, the statistical significance of
250 the effects of warming, grazing and their interaction on plant biomass, soil properties,
251 microbial functional genes, and nitrification and denitrification enzyme activity from
252 bacteria and fungi were tested by two-way ANOVA in the PROC GLM procedure of
253 SAS (version 9, SAS Institute, Cary, NC, USA).

254

255 **3 Results**

256 **3.1 Plant biomass and soil properties**

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

260 Soil temperature varied from 11.8 to 14.0 ^{o}C. Grazing ($P=0.05$) and warming ($P<0.01$)

increased soil temperature. 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). 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). Similar to TC, soil total N (TN) also showed no response to grazing or warming (Fig. 2b). Soil NH_4^+-N content was lower in warming treatments than in no-warming treatments ($P=0.05$) (Fig. 2c). Greater soil NO_3-N content occurred under the warming treatments ($P=0.05$) than under the no-warming treatments (Fig. 2d).

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. By comparison, fungal gene abundance showed no difference across all treatments.

3.3 Nitrification and denitrification enzyme activity from bacteria and fungi

Total nitrification enzyme activity (TNEA) varied from 1.07 to 1.64 μg N g^{-1} h^{-1} in all treatments. Bacterial nitrification enzyme activity (BNEA) ranged from 0.43 to 0.64 μg g^{-1} h^{-1}, which was lower than the fungal nitrification enzyme activity (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).

Total denitrification enzyme activity (TDEA) was between 1.32 and 1.80 μg N g^{-1} h^{-1}. Fungal denitrification enzyme activity (FDEA) was clearly the dominant process for TDEA (Fig. 4 d-f), because it was higher than bacterial denitrification enzyme activity (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).

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 the contribution of FNEA and FDEA to TNEA and TDEA in soils (FNEA: P=0.02; FDEA: P=0.04). There were no differences in the contribution of FNEA and FDEA to TNEA and TDEA in any treatments.

4 Discussion

N_2O was mainly produced from the microbial nitrification and denitrification processes, but the microbial pathway of these processes was still unclear. In our results, fungi contributed 54% and 63% of the TNEA and TDEA, respectively, in the alpine grassland studied. Our result of the fungal contribution to N_2O production is much lower than Laughlin and Stevens (2002) and Zhong *et al.* (2018) who reported 89% and 86% fungal contribution from temperate grasslands, but is higher than the 40-51% fungal contribution observed across different ecosystems by Chen et al. (2014). Kato et

al. (2013) showed that N_2O emissions from FDEA was higher than from BDEA in alpine meadows, reinforcing the important role fungi play in the N_2O production process. Our findings support our first hypothesis and further proved that both denitrification and nitrification were largely driven by fungal communities in alpine grasslands. A possible explanation is that fungi prefer the arid, high complex compounds subtract substrate and low-temperature environment (Pietikäinen et al., 2005;Chen et al., 2015;Marusenko et al., 2013). In alpine grasslands, the mean annual temperature is 0 ^{o}C; even during the sampling day the mean temperature was only 11 ^{o}C. The cold environment could cause higher activity in fungi than in bacteria. Moreover, the cold environment decreases the rate of mineralization, leading to greater 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). Inorganic C and inorganic N content were also much higher than study in temperate grasslands (Zhong et al. 2018), but lower than temperate farmland ecosystems (Chen et al., 2015;Laughlin and Stevens, 2002); this is mainly because the fungal contribution to N_2O potential and N loss in the alpine grasslands was lower than in temperate grasslands but higher than farmland on the Qinghai-Tibetan Plateau.

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 common 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 producing 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 our second hypothesis that although warming did not change the potential N_2O emissions on the Qinghai-Tibetan Plateau, the biotic pathways responsible for N_2O had been changed, as bacterial contribution to N_2O potential was higher than fungal under the warming treatment (Fig. 4). The increase in bacterial N_2O production potential, coupled with decrease in fungal N_2O production, could be the main reason why there was no difference between control and warming treatments. The field data in our site was measured in year of 2011–2012 and also showed no effect of warming on N_2O emission (Zhu et al. 2015). Our results reinforced this and suggested that bacterial nitrification and denitrification alone is unable to accurately describe the response of N_2O to warming. It is the two reasons that lead to the changes of fungal and bacterial pathways for N_2O emissions by warming. Firstly, the increased of soil temperature directly reduce fungal activity but increase bacterial activity, because fungi prefer the low-temperature environment environment compared with bacteria. Secondly, warming indirectly reduce fungal activity but increase bacterial activity

through increased soil inorganic N and decreased soil organic N, because fungi prefer higher organic C/N environment while bacteria prefer higher inorganic C/N environment. In our site, although the soil NH_4^+-N concentration did not change with warming, soil NO_3^--N concentration was significantly increased showed the soil inorganic N was increased (Fig. 2a and 2b); on the other hand, the soil dissolved organic nitrogen was significantly decreased from 48 to 41 mg kg^{-1} ($P<0.04$), the soil labile C and N was also found significantly decreased by warming (Rui et al., 2012), it showed the soil organic C and N was decreased in our site. All these changes could directly and indirectly inhibit the growth of fungal communities and their activity, but increase those of bacteria. Although the gene abundance of fungi was not changed, the FNEA and FDEA were reduced by 16% and 30% respectively by warming, and BDEA was increased by 41%. All these changes resulted in fungi contributing less to nitrification and denitrification than bacteria (Fig.5). This indicates that the soil microbial process was altered by warming, even though the TNEA and TDEA did not change, with a shift in the dominance from fungi to bacteria on N_2O production 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, in this study

fungal and bacterial nitrification and denitrification activity showed little response to winter grazing. A possible explanation is that neither soil moisture, plant biomass nor organic/inorganic C/N content were affected by winter grazing (Fig.1-2). 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 (Zhu et al., 2015; Krümmelbein et al., 2009). As a result, the same soil environmental conditions for both winter grazing and control had no effect on soil fungi and bacteria, and thus on fungal and bacterial nitrification and denitrification. Moreover, the field data of N_2O emission in the year of 2011-2012 also supports the results of Zhu et al. (2015) and suggests that replacing summer grazing by winter grazing could cause the soil N cycle process to become stable.

Overall, we conclude that fungi played the dominant role in the soil N cycle, and could be the major source of N_2O production and N loss in alpine meadows. Climate warming is not likely to affect potential N_2O emissions but could alter biotic pathways responsible for N_2O production on the Tibetan Plateau. Our study exhibited the effects of a decade of simulation experiment; however, a thorough understanding about the long-term impact of warming and grazing on soil fungal nitrification and denitrification from alpine meadow grassland requires further investigation for multi-decade period.

From this study, due to the different adaptation strategies of fungi and bacteria, and their different nutrition requirements, future changes in climate and soil resources are likely to affect biogeochemistry in a way not currently accounted for in ecosystem models that assume N transformations are controlled only by bacteria. Accurate predictions for N_2O production and N loss due to environmental change and land use

will benefit from the inclusion of fungi as key mediators of ecological processes in grasslands.

Competing interests

The authors declare that they have no conflict of interest.

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554 **Figure caption**

555

556 **Fig. 1**, Plant biomass (a) soil temperature (b) and soil moisture content (c) in an alpine
557 meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming
558 treatment; WG (▨), warming combined with the winter grazing treatment. Values are
559 means ±1 s.e.m. (*n*=4). Different letters indicate significant differences within each
560 treatment (P<0.05).
561

562

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

567

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

571

572 **Fig. 4** Bacterial nitrification enzyme activity (BNEA) (a), fungal nitrification enzyme
573 activity (FNEA) (b), total nitrification enzyme activity (TNEA) (c); Bacterial
574 denitrification enzyme activity (BDEA) (d), fungal denitrification enzyme activity
575 (FDEA) (e) and total denitrification enzyme activity (TDEA) (f) in an alpine meadow.
576 C(■), control treatment; G (□), winter grazing treatment; W (▩), warming treatment;
577 WG (▨), warming combined with the winter grazing treatment. Values are means ±1
578 s.e.m. (*n*=4). Different letters indicate significant differences within each treatment
579 (P<0.05).
580

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

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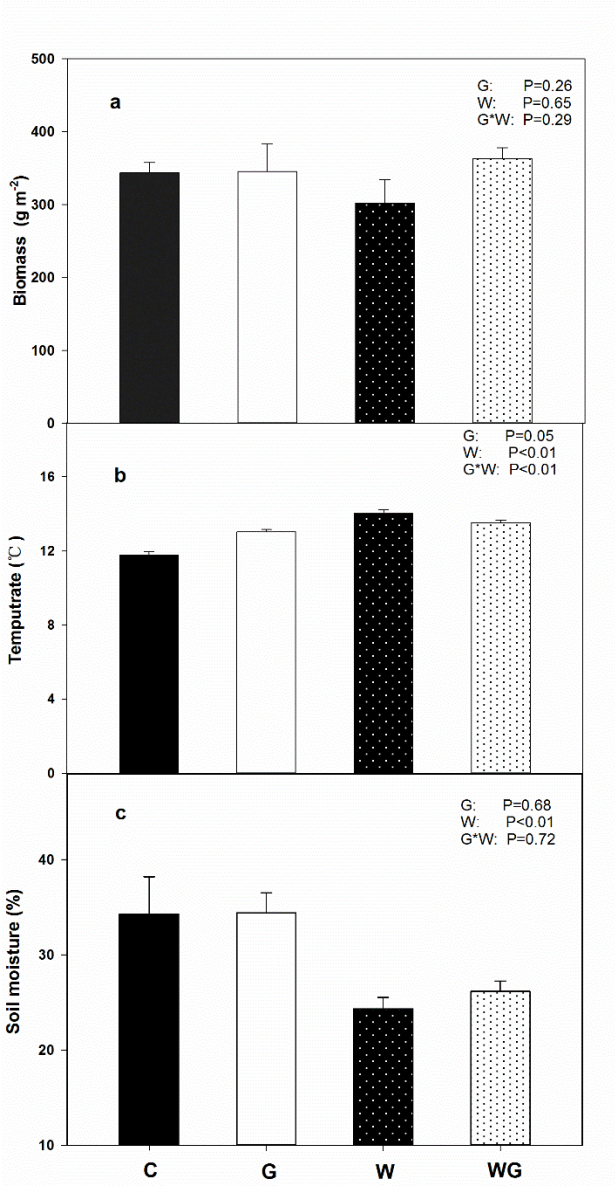
590 Fig.1



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593 Fig. 2

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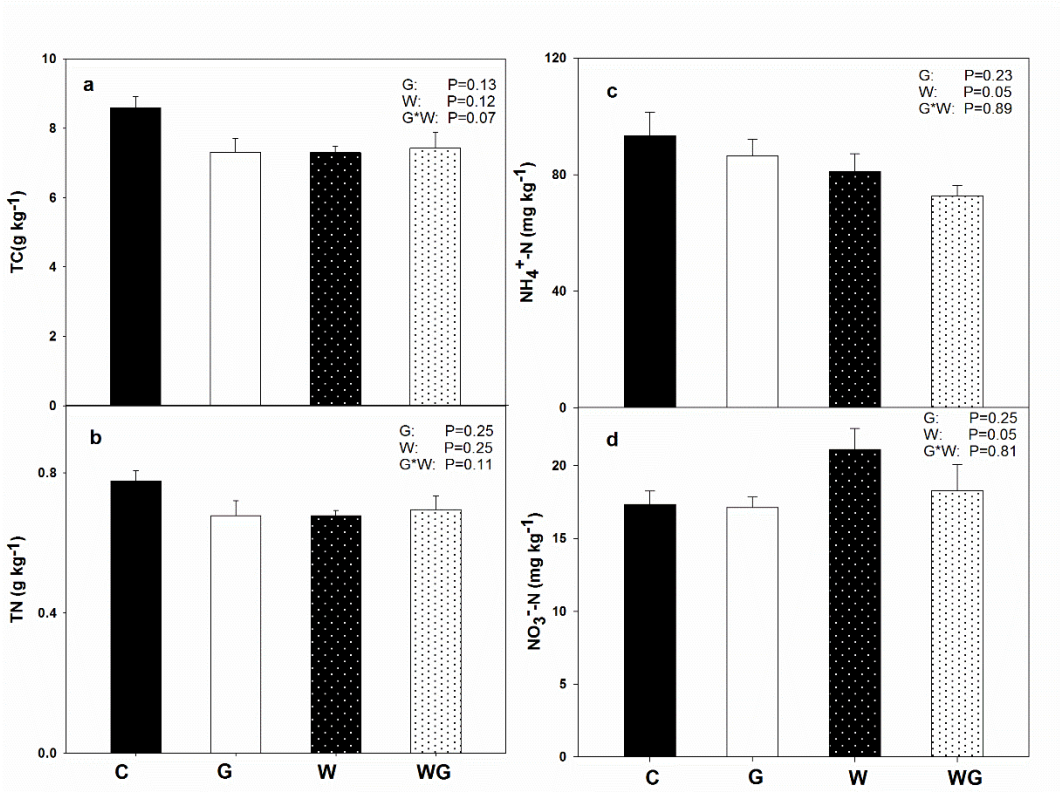

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598 Fig. 3

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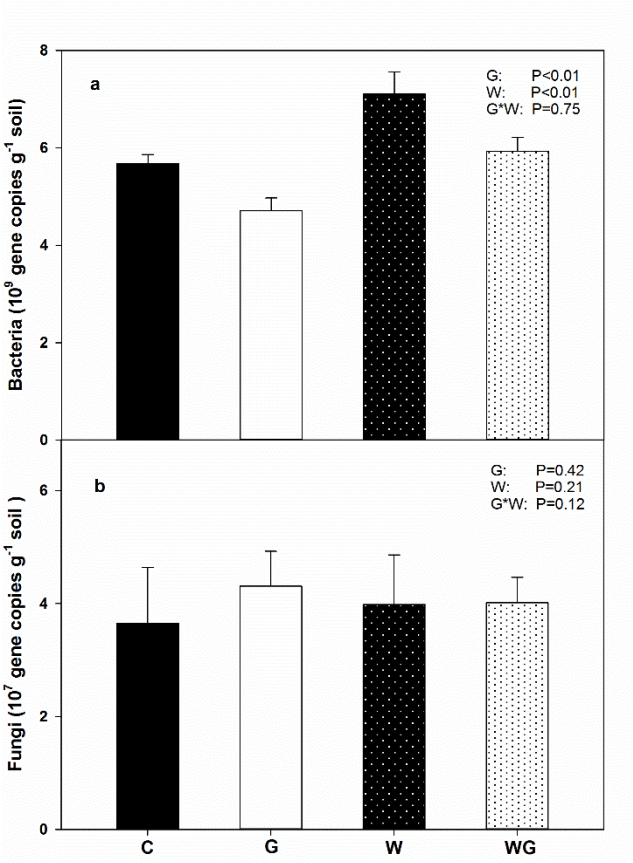

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605 Fig.4

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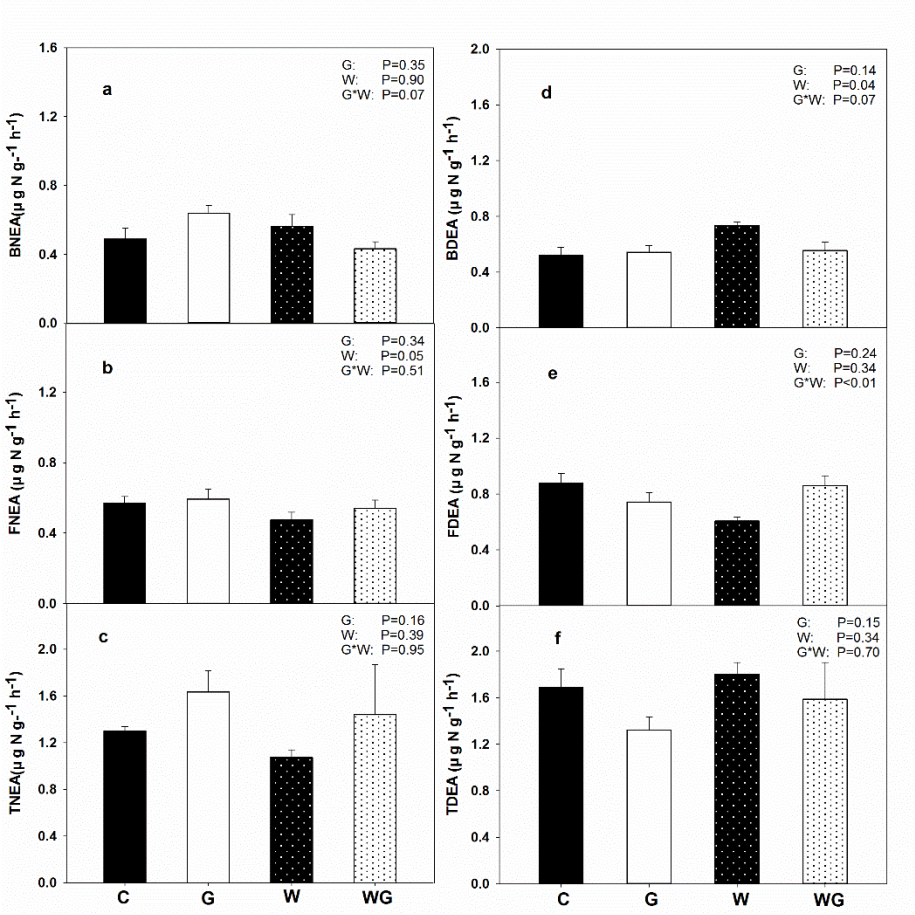

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609 Fig.5

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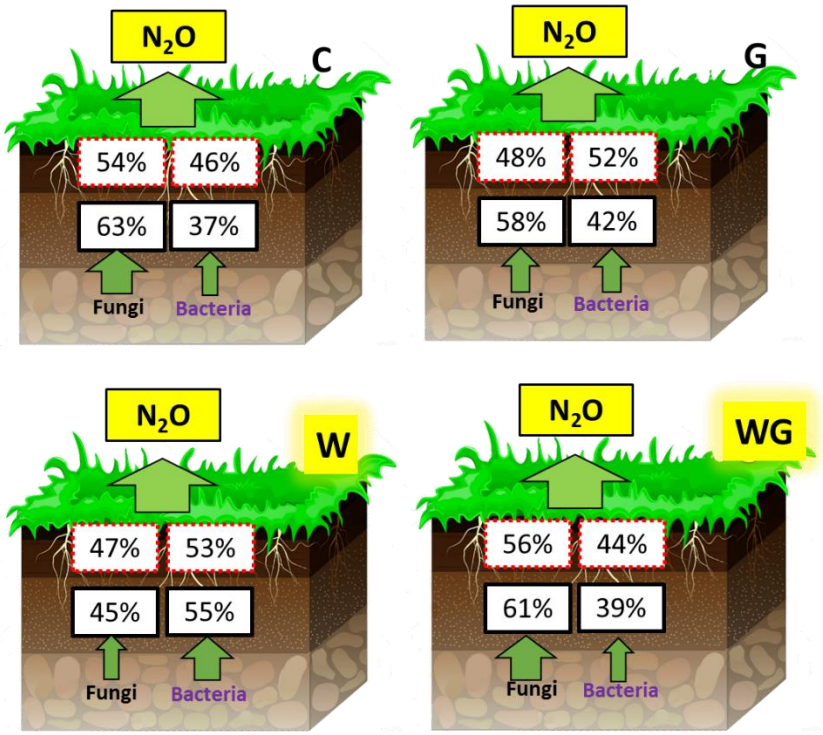

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