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

Lei Zhong[1], Shiping Wang[2], Xingliang Xu[3], Yanfen Wang[4], Yichao Rui[5], Xiaoqi Zhou[6], Qinhua Shen[7], Jinzhi Wang[8], Lili Jiang[2], Caiyun Luo[9], Tianbao Gu[1], Wenchao Ma[1], Guanyi Chen[1, 10]

[1]School of Environmental Science and Engineering, Tianjin University / China-Australia Centre for Sustainable Urban Development, Tianjin 300350, China
[2] Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau Research, Chinese Academy of Sciences, Beijing 100101, China
[3]Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources, Chinese Academy of Sciences, Beijing 100101, China
[4] University of Chinese Academy of Sciences, Beijing 100049, China
[5] Department of Soil Science, University of Wisconsin-Madison, Madison, WI 53706, USA
[6] Tiantong National Forest Ecosystem Observation and Research Station, Center for Global Change and Ecological Forecasting, School of Ecological and Environmental Sciences, East China Normal University, Shanghai 200241, China
[7] Institute of Agriculture and Environment, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand.
[8] Beijing Key Laboratory of Wetland Services and Restoration, Institute of Wetland Research, Chinese Academy of Forestry, Beijing 100091, China
[9] Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
[10]School of Science, Tibet University, No. 36 Jiangsu Street, Lhasa 850012, Tibet Autonomous Region, China

Author for correspondence:
Dr. Wenchao Ma；Prof. Guanyi Chen
School of Environmental Science and Engineering, Tianjin University / China-Australia Centre for Sustainable Urban Development, Tianjin 300072, China
Email: mawc916@tju.edu.cn；chenguanyi@utibet.edu.cn

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 for 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

62 ecosystem nitrogen cycle model.

63

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

65

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 are 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

88 grazing from October to May (Cui et al., 2014), which host about 13.3 million domestic
89 yaks and 50 million sheep, with dramatically increasing numbers in future (Yao et al.,
90 2006). Grazing strongly affects soil N cycling, as well as plant and microbial diversity
91 (Hillebrand, 2008) and the stability of ecosystems (Klein et al., 2004). Previous studies
92 have demonstrated losses of N caused by warming (Klein et al., 2004; 2007) and that
93 overgrazing (Zhou et al., 2005) leads to degradation in alpine grasslands. The effects of
94 climate warming and grazing on the aboveground vegetation, soil physicochemical
95 properties, litter mass loss, bacterial communities and N_2O fluxes of Tibetan alpine
96 grasslands have been extensively investigated (Hu et al., 2010;Li et al., 2016;Luo et al.,
97 2010;Rui et al., 2012;Wang et al., 2012;Zhu et al., 2015); however, most of these studies
98 was focused on the effect of summer grazing, little is showed the effect of winter
99 grazing on them (Zhu et al. 2015; Che et al. 2018). On the other hand, many studies of
100 Tibetan alpine grasslands are mainly focused on bacterial nitrifiers and denitrifiers or
101 their activities, taking these to be the key factors on N_2O emission in alpine grasslands.
102 However, while many studies have explored N mineralization, nitrification and even
103 denitrification as well as bacterial nitrifiers and denitrifiers for better understanding of
104 N_2O emission and ecosystem functioning (Yang et al., 2013;Yue et al., 2015), few
105 studies have been conducted to distinguish whether bacteria or fungi dominate in N_2O
106 emission and N cycling (Kato et al., 2013), especially under warming and grazing
107 conditions.

108 Since optimum environments for fungi and bacteria are different, they may respond
109 differently to environmental changes. Fungi prefer a 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 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 soil sampling year and month of 2015, mean temperature was 0 ^{o}C and 9.7 ^{o}C, respectively; total rainfall was 327.2 mm and 46.6 mm, respectively. Over 80% of total rainfall falls during the summer monsoon season (Luo et al., 2010; Zhao and Zhou, 1999). The soil was classified as Gelic Cambisols (WRB, 1998). 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 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

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 to make sure the
157 increased of soil temperature was the same with it in summer, as some infrared
158 thermometers were not working.

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

175 **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). Total C and N content were measured by using combustion elemental analyzers (PerkinElmer, EA2400, USA).

Soil DNA was extracted from 0.5 g of 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 ºC, 30 s at 62 ºC and 30 s at 72 ºC. A final 5-min extension step completed the protocol.

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

Fungal (FNEA), bacterial (BNEA) and total nitrification enzyme activity (TNEA) were 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 slurry 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). 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 was 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 soil ($P<0.01$), 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 N g^{-1} h^{-1}, which was lower than the FNEA in soil (0.59–0.66 μg N 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

303 the contribution of FNEA and FDEA to TNEA and TDEA in soils (FNEA: P=0.02;
304 FDEA: P=0.04).
305
306 **4 Discussion**
307 N_2O is mainly produced from 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 were higher than from BDEA in
316 alpine meadows, reinforcing the important role fungi play in the 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.,
325 1998;Schmidt et al., 2004). In our study, soil TC and TN concentrations were 72–86 g

326 kg^{-1} and 6–7 g kg^{-1}, respectively (Fig. 2a and 2b), much higher than in temperate
327 grasslands and farmland, providing a favorable environment for fungi (Bai et al., 2010).
328 These are mainly reasons that soil fungi played the mainly role in N_2O production
329 process in the Tibetan alpine grasslands.

330 Our methodology did not exclude a role for archaea in nitrification and denitrification.
331 Previous studies on grasslands only focused on fungal and bacterial process because
332 archaeal specific inhibitors have not yet been identified for N cycling processes.
333 However, archaea are widespread in soil, are involved in nitrification denitrification
334 (Cabello et al., 2004), eg. archaeal ammonia oxidizers are globally (Leininger et al.,
335 2006). In our study, we also found the TNEA was higher than the sum of NEA from
336 bacteria and fungi, while TDEA was higher than DEA from bacteria and fungi (Fig. 4),
337 which showed that archaea also played the role in N_2O production process in our site.
338 The development of inhibitor-based approaches may help to show how archaea
339 responses to environmental change (Marusenko et al. 2013).

340 Our results supported the second hypothesis that although warming did not change
341 the total N_2O production potential on the Qinghai-Tibetan Plateau, the biotic pathways
342 responsible for N_2O had been changed, as bacterial contribution to TNEA and TDEA
343 all were higher than fungal which suggested the higher bacterial N_2O production
344 potential under warming treatment (Fig. 4, Table 1). The increase in bacterial N_2O
345 production potential, coupled with a decrease in fungal N_2O production potential, could
346 be the main reasons why the total N_2O production potential was no difference between
347 control and warming treatments. The field data of N_2O emission in our site was

348 measured in the year of 2011–2012 also showed no effect of warming on N_2O emission
349 (Zhu et al. 2015). Our results reinforced this and suggested that bacterial nitrification
350 and denitrification process alone is unable to accurately describe the response of N_2O
351 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 reduces 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 were 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
369 fungi was not changed by warming which showed inconsistencies with the changes of

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

378 Numerous studies have demonstrated that grazing can impact microbial processes
379 and induce the loss of N through: (1) altering the substrate concentration for N_2O
380 production and reduction in soil through the deposition of dung and urine (Saggar et al.,
381 2004); (2) reducing vegetation cover due to changes in soil water content and energy
382 balance (Leriche et al., 2001); and (3) increasing soil compaction and reducing soil
383 aeration through animal tramping (Houlbrooke et al., 2008). However, most of these
384 were focused on grazing in the growing season, little was focused on the effect of winter
385 grazing on N cycle process. In this study fungal and bacterial potential of N_2O
386 production from nitrification and denitrification all showed little response to winter
387 grazing (Fig. 4, Table 1). A possible explanation is that neither soil moisture, plant
388 biomass nor organic/inorganic C/N content was affected by winter grazing (Fig.1-2,
389 Table 1). Additionally, the soil was frozen in winter, so that the effect of selective
390 feeding and trampling could be limited by grazing sheep (Zhu et al., 2015;
391 Krümmelbein et al., 2009; Steffens et al., 2008). 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 support the results and suggest that replacing summer grazing by winter grazing could cause the soil N cycle process to become stable (Zhu et al. 2015).

Overall, we conclude that fungi played the dominant role in the N_2O production process in alpine meadows. Previous study had proved the climate warming did not affect the N_2O production in our site (Zhu et al. 2015), but we found warming could alter biotic pathways responsible for N_2O production process on the Tibetan Plateau. Our study exhibited the effects of a decade of the 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 a 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.

Acknowledgements

This work was supported by the National Key R&D Program of China (No. 2016YFC0501802), the National Natural Science Foundation of China (No. 41601245; 31672474), the Foundation of Committee on Science and Technology of Tianjin (No. 16YFXTSF00500), and supported by the Strategic Priority Research Program B of the Chinese Academy of Sciences (No. XDB15010201). We also thank Miss Ri Weal for her assistance in improving the use of English in the manuscript.

References

Bai, Y., Jianguo, W. U., Clark, C. M., Naeem, S., Pan, Q., Huang, J., Zhang, L., and Han, X.: Tradeoffs and thresholds in the effects of nitrogen addition on biodiversity and ecosystem functioning: evidence from inner Mongolia Grasslands, Global Change Biol., 16, 358-372, 2010.

Borneman, J., and Hartin, R. J.: PCR primers that amplify fungal rRNA genes from environmental samples, Appl. Environ. Microb., 66, 4356, 2000.

Cabello, P., Roldán, M.D., Moreno-Vivián, C.: Nitrate reduction and the nitrogen cycle in archaea. Microbiol. 150 (11), 3527 – 3546, 2004.

Cao, G., Xu, X., Long, R., Wang, Q., Wang, C., Du, Y., and Zhao, X.: Methane emissions by alpine plant communities in the Qinghai–Tibet Plateau, Biol. Letters, 4, 681-684, 2008.

Castaldi, S., and Smith, K. A.: Effect of cycloheximide on N_2O and NO_3^- production in a forest and an agricultural soil, Biol. Fert. Soils, 27, 27-34, 1998.

Che, R., Deng, Y., Wang, W., Rui, Y., Zhang, J., Tahmasbian, I., Tang, L., Wang, S., Wang Y., Xu, Z., and Cui, X.: Long-term warming rather than grazing significantly changed total and active soil procaryotic community structures, Geoderma, 316: 1-10, 2018.

Chen, H., Mothapo, N. V., and Shi, W.: The significant contribution of fungi to soil N_2O production across diverse ecosystems, Appl. Soil Ecol., 73, 70-77, 2014.

Chen, H., Mothapo, N. V., and Shi, W.: Fungal and bacterial N_2O production regulated by soil amendments of simple and complex substrates, Soil Biol. Biochem., 84, 116-126, 10.1016/j.soilbio.2015.02.018, 2015.

Cui, S., Zhu, X., Wang, S., Zhang, Z., Xu, B., Luo, C., Zhao, L., and Zhao, X.: Effects of seasonal grazing on soil respiration in alpine meadow on the Tibetan plateau, Soil Use Manage., 30, 435-443, 2015.

Dassonville, N., Guillaumaud, N., Piola, F., Meerts, P., and Poly, F.: Niche construction by the invasive Asian knotweeds (species complex Fallopia): Impact on activity, abundance and community structure of denitrifiers and nitrifiers, Biol. Invasions, 13, 1115-1133, 2011.

Giorgi, F., Whetton, P. H., Jones, R. G., Christensen, J. H., Mearns, L. O., Hewitson, B., Vonstorch, H., Francisco, R., and Jack, C.: Emerging patterns of simulated regional climatic changes for the 21st century due to anthropogenic forcings, Geophys. Res. Lett., 28, 3317-3320, 2001.

Hayatsu, M., Tago, K., and Saito, M.: Various players in the nitrogen cycle: Diversity and functions of the microorganisms involved in nitrification and denitrification, J. Soil Sci. Plant Nut., 54, 33-45, 10.1111/j.1747-0765.2007.00195.x, 2008.

Hillebrand, H.: Grazing regulates the spatial variability of periphyton biomass, Ecology, 89, 165-173, 2008.

Houlbrooke, D. J., Littlejohn, R. P., Morton, J. D., and Paton, R. J.: Effect of irrigation and grazing animals on soil quality measurements in the North Otago Rolling Downlands of New Zealand, Soil Use Manage., 24, 416–423, 2008.

Hu, Y., Chang, X., Lin, X., Wang, Y., Wang, S., Duan, J., Zhang, Z., Yang, X., Luo, C., and Xu, G.: Effects of warming and grazing on N_2O fluxes in an alpine meadow ecosystem on the Tibetan plateau, Soil Biol. Biochem., 42, 944-952, 2010.

Huang, Y., Xiao, X., and Long, X.: Fungal denitrification contributes significantly to N_2O production in a highly acidic tea soil, J. Soil Sediment., 17, 1599-1606, 10.1007/s11368-017-1655-y, 2017.

Ineson, P., Benham, D. G., Poskitt, J., Harrison, A. F., Taylor, K., and Woods, C.: Effects of climate change on nitrogen dynamics in upland soils. 2. A soil warming study, Global Change Biol., 4, 143-152, 1998.

Kato, T., Toyoda, S., Yoshida, N., Tang, Y., and Wada, E.: Isotopomer and isotopologue signatures of N_2O produced in alpine ecosystems on the Qinghai-Tibetan Plateau, Rapid Commun. Mass sp., 27, 1517-1526, 2013.

Kimball, B.A., Conley, M.M., Wang, S.P., Lin, X.W., Luo, C.Y., Morgan, J., Smith, D.: Infrared heater arrays for warming ecosystem field plots. Global Change Biol., 14, 309–320, 2008.

Klein, J. A., Harte, J., and Zhao, X. Q.: Experimental warming causes large and rapid species loss, dampened by simulated grazing, on the Tibetan Plateau, Ecol. Lett., 7, 1170-1179, 2004.

Klein, J. A., Harte, J., and Zhao, X. Q.: Experimental warming, not grazing, decreases rangeland quality on the Tibetan Plateau, Ecol. Appl., 17, 541, 2007.

Klotz, M. G., and Stein, L. Y.: Nitrifier genomics and evolution of the nitrogen cycle, Fems Microbiol. Lett., 278, 146-156, 10.1111/j.1574-6968.2007.00970.x, 2008.

Krümmelbein, J., Peth, S., Zhao, Y., Horn, R.:. Grazing‐induced alterations of soil hydraulic properties and functions in Inner Mongolia, PR China. J. Plant Nutr. Soil Sc., 172(6), 769-776, 2009.

Laughlin, R. J., and Stevens, R. J.: Evidence for fungal dominance of denitrification and codenitrification in a grassland soil, Soil Sci. Soc. Am. J., 66, 1540-1548, 2002.
Laughlin, R. J., Rutting, T., Mueller, C., Watson, C. J., and Stevens, R. J.: Effect of acetate on soil respiration, N2O emissions and gross N transformations related to fungi and bacteria in a grassland soil, Appl. Soil Ecol., 42, 25-30, 10.1016/j.apsoil.2009.01.004, 2009.
LeBauer, D. S., and Treseder, K. K.: Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed, Ecology, 89, 371-379, 10.1890/06-2057.1, 2008.
Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., Prosser, J.I., Schuster, S.C., Schleper, C.: Archaea predominate among ammoniaoxidizing prokaryotes in soils. Nature, 442, 806-809, 2006.
Leriche, H., Leroux, X., Gignoux, J., Tuzet, A., Fritz, H., Abbadie, L., and Loreau, M.: Which functional processes control the short-term effect of grazing on net primary production in grasslands?, Oecologia, 129, 114-124, 2001.
Li, Y., Lin, Q., Wang, S., Li, X., Liu, W., Luo, C., Zhang, Z., Zhu, X., Jiang, L., and Li, X.: Soil bacterial community responses to warming and grazing in a Tibetan alpine meadow, Fems Microbiol. Ecol., 92, fiv152, 2016.
Luo, C., Xu, G., Chao, Z., Wang, S., Lin, X., Hu, Y., Zhang, Z., Duan, J., Chang, X., and Su, A.: Effect of warming and grazing on litter mass loss and temperature sensitivity of litter and dung mass loss on the Tibetan plateau, Global Change Biol., 16, 1606-1617, 2010.
Marusenko, Y., Huber, D. P., and Hall, S. J.: Fungi mediate nitrous oxide production but not ammonia oxidation in aridland soils of the southwestern US, Soil Biol. Biochem., 63, 24-36, 2013.
Muyzer, G., Waal, E. C. D., and Uitterlinden, A. G.: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environmen. Microbiol., 59, 695, 1993.
Patra, A. K., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S. J., Guillaumaud, N., Loiseau, P., Louault, F., Mahmood, S., and Nazaret, S.: Effects of management regime and plant species on the enzyme activity and genetic structure of N‐fixing, denitrifying and nitrifying bacterial communities in grassland soils, Environ. Microbiol., 8, 1005-1016, 2006.
Pietikäinen, J., Pettersson, M., and Bååth, E.: Comparison of temperature effects on soil respiration and bacterial and fungal growth rates, Fems Microbiol. Ecol., 52, 49, 2005.
Rui, Y., Wang, Y., Chen, C., Zhou, X., Wang, S., Xu, Z., Duan, J., Kang, X., Lu, S., and Luo, C.: Warming and grazing increase mineralization of organic P in an alpine meadow ecosystem of Qinghai-Tibet Plateau, China, Plant Soil, 357, 73-87, 2012.
Saggar, S., Bolan, N. S., Bhandral, R., Hedley, C. B., and Luo, J.: A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures, New Zeal. J. Agr. Res., 47, 513-544, 2004.
Schmidt, I. K., Tietema, A., Williams, D., Gundersen, P., Beier, C., Emmett, B. A., and

536 Estiarte, M.: Soil Solution Chemistry and Element Fluxes in Three European
537 Heathlands and Their Responses to Warming and Drought, Ecosystems, 7, 638-649,
538 2004.
539 Shi, H., Hou, L., Yang, L., Wu, D., Zhang, L., and Li, L.: Effects of grazing on CO_2,
540 CH_4, and N_2O fluxes in three temperate steppe ecosystems, Ecosphere, 8, e01760,
541 2017.
542 Steffens, M., Kölbl, A., Totsche, K.U., Kögel-Knabner, I.: Grazing effects on soil
543 chemical and physical properties in a semiarid steppe of Inner Mongolia (PR China),
544 Geoderma, 143(1-2): 63-72, 2008.
545 Thompson, L. G., Mosley-Thompson, E., Davis, M., Lin, P. N., Yao, T., Dyurgerov,
546 M., and Dai, J.: "Recent warming": ice core evidence from tropical ice cores with
547 emphasis on Central Asia, Global Planet. Change, 7, 145-156, 1993.
548 Thompson, L. G.: Ice core evidence for climate change in the Tropics: implications for
549 our future, Quaternary Sci. Rev., 19, 19-35, 2000.
550 Tian, H., Wang, S., Liu, J., Pan, S., Chen, H., Zhang, C., and Shi, X.: Patterns of soil
551 nitrogen storage in China, Global Biogeochem. Cy., 20, 247-247, 2006.
552 Wang, B., and French, H. M.: Climate controls and high‐altitude permafrost,
553 qinghai‐xizang (tibet) Plateau, China, Permafrost Periglac., 5, 87-100, 1994.
554 Wang, S., Duan, J., Xu, G., Wang, Y., Zhang, Z., Rui, Y., Luo, C., Xu, B., Zhu, X., and
555 Chang, X.: Effects of warming and grazing on soil N availability, species
556 composition, and ANPP in an alpine meadow, Ecology, 93, 2365-2376, 2012.
557 WRB, 1998. World Reference Base for Soil Resources. FAO/ISRIC/ISSS, Italy.
558 Yang, Y., Wu, L., Lin, Q., Yuan, M., Xu, D., Yu, H., Hu, Y., Duan, J., Li, X., and He,
559 Z.: Responses of the functional structure of soil microbial community to livestock
560 grazing in the Tibetan alpine grassland, Global change biol., 19, 637-648, 2013.
561 Yao, J., Yang, B., and Yan, P.: Analysis on habitat variance and behaviour of Bos
562 gruiens in China, Acta Prataculturae Sinica, 15, 124-128, 2006. (In Chinese)
563 Yue, H., Wang, M., Wang, S., Gilbert, J. A., Sun, X., Wu, L., Lin, Q., Hu, Y., Li, X.,
564 and He, Z.: The microbe-mediated mechanisms affecting topsoil carbon stock in
565 Tibetan grasslands, ISME J., 9, 2012, 2015.
566 Zhao, X. Q., and Zhou, X. M.: Ecological Basis of Alpine Meadow Ecosystem
567 Management in Tibet: Haibei Alpine Meadow Ecosystem Research Station, 28, 642-
568 647, 1999.
569 Zheng, D., Zhang, Q., and Wu, S.: Mountain Geoecology and Sustainable Development
570 of the Tibetan Plateau, Geojournal Library, 57, 2000.
571 Zhong, L., Bowatte, S., Newton, P. C., Hoogendoorn, C. J., Luo, D.: An increased ratio
572 of fungi to bacteria indicates greater potential for N_2O production in a grazed
573 grassland exposed to elevated CO_2. Agr. Ecol. Environ. 254, 111-116, 2018.
574 Zhou, H., Zhao, X., Tang, Y., Gu, S., and Zhou, L.: Alpine grassland degradation and
575 its control in the source region of the Yangtze and Yellow Rivers, China, Grassl. Sci.,
576 51, 191–203, 2005.
577 Zhu, X., Luo, C., Wang, S., Zhang, Z., Cui, S., Bao, X., Jiang, L., Li, Y., Li, X., and
578 Wang, Q.: Effects of warming, grazing/cutting and nitrogen fertilization on
579 greenhouse gas fluxes during growing seasons in an alpine meadow on the Tibetan

Plateau, Agr. Forest Meteorol., 214, 506-514, 2015.
Zumft, W. G.: Cell biology and molecular basis of denitrification, Microbiol. Mol. Biol. R., 61, 533-616, 1997.

584 Table 1. Results (F-value and P-value) from two-way ANOVA for the effects of
585 warming (W), winter grazing (G) and their interactions (WG) on soil and microbial
586 characteristics.
587
588
589

	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
Bacteria	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 $P < 0.05$.

590

591 **Figure caption**

592

593 **Fig. 1**, Plant biomass (a) soil temperature (b) and soil moisture content (c) in an alpine
594 meadow. C (■), control treatment; G (□), winter grazing treatment; W (▩), warming
595 treatment; WG (▨), warming combined with the winter grazing treatment. Values are
596 means ±1 s.e.m. (*n*=4).
597

598

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

603

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

607

608 **Fig. 4** Bacterial nitrification enzyme activity (BNEA) (a), fungal nitrification enzyme
609 activity (FNEA) (b), total nitrification enzyme activity (TNEA) (c); Bacterial potential
610 of N_2O production from denitrification (BDEA) (d), fungal potential of N_2O production
611 from denitrification (FDEA) (e) and total potential of N_2O production from
612 denitrification (TDEA) (f) in an alpine meadow. C (■), control treatment; G (□),
613 winter grazing treatment; W (▩), warming treatment; WG (▨), warming combined
614 with the winter grazing treatment. Values are means ±1 s.e.m. (*n*=4).
615

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

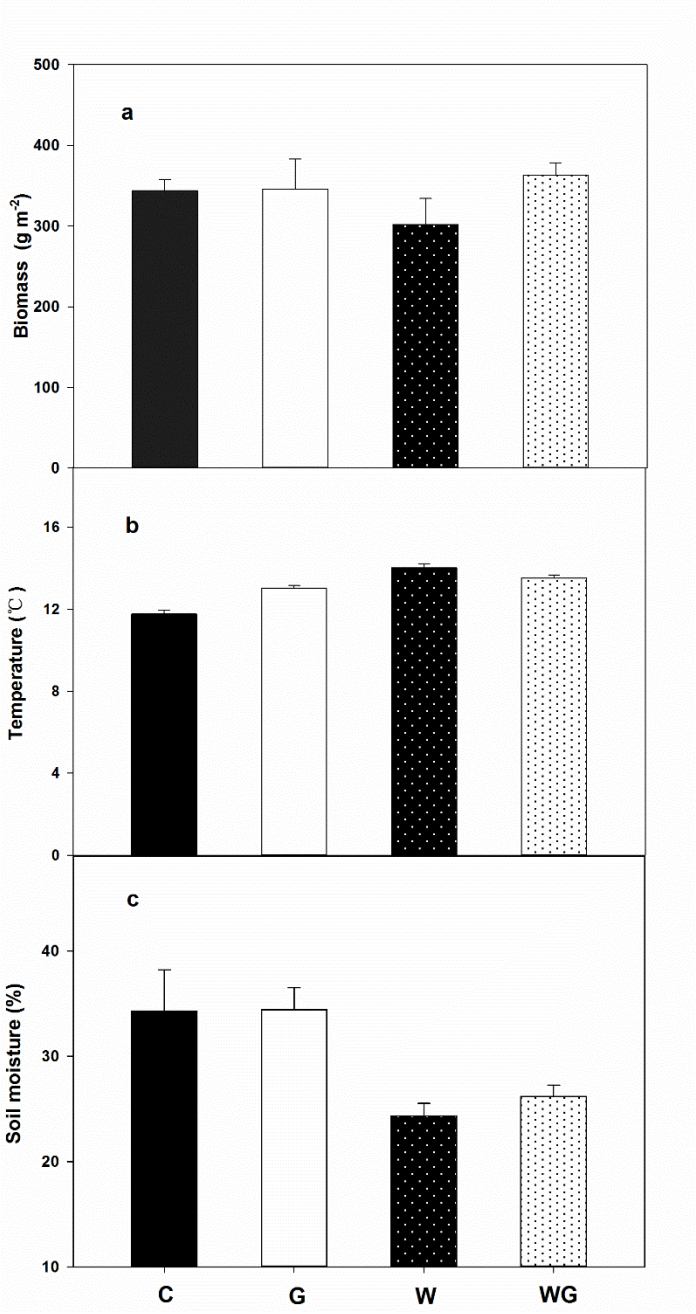
621

622

623

624

625 Fig.1



626

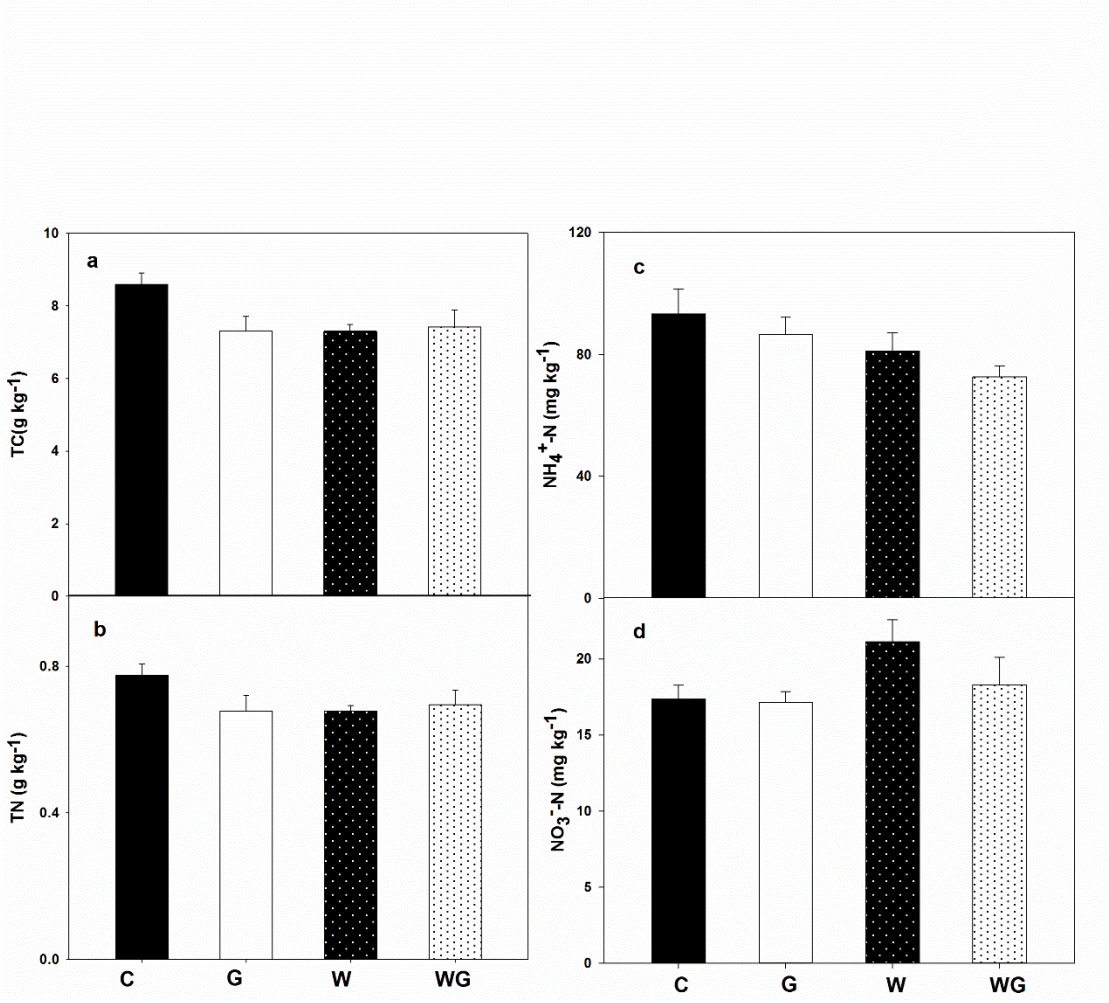
627

628 Fig. 2

629

630

631



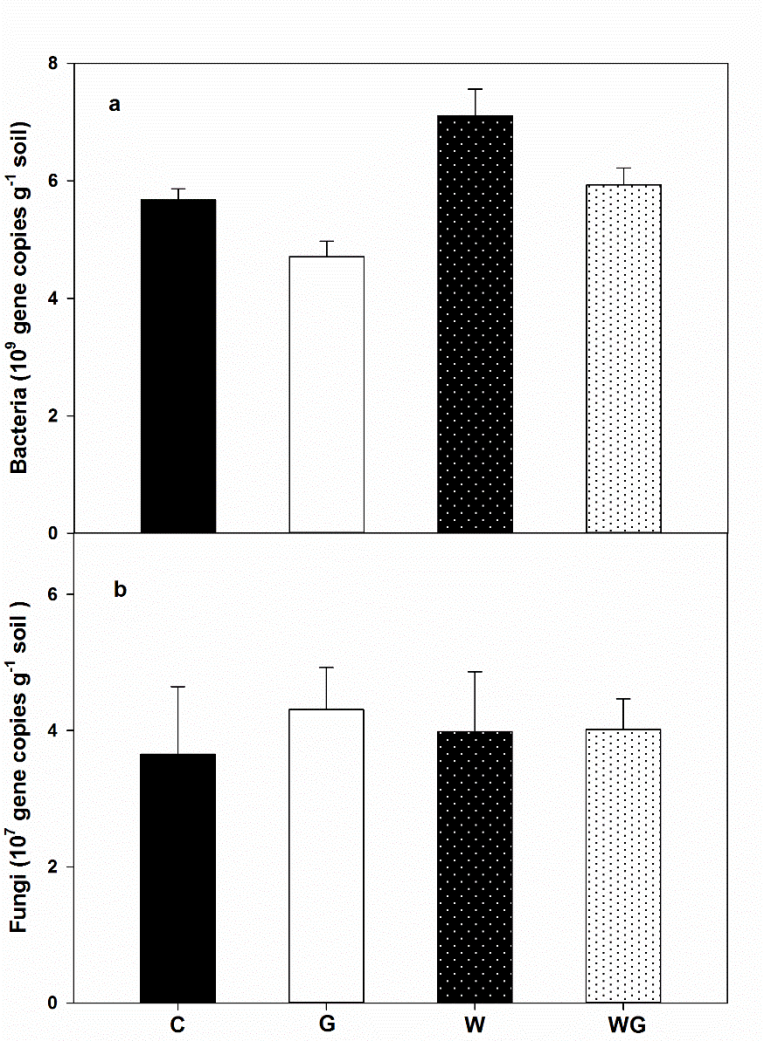
632

633 Fig. 3

634

635

636



637

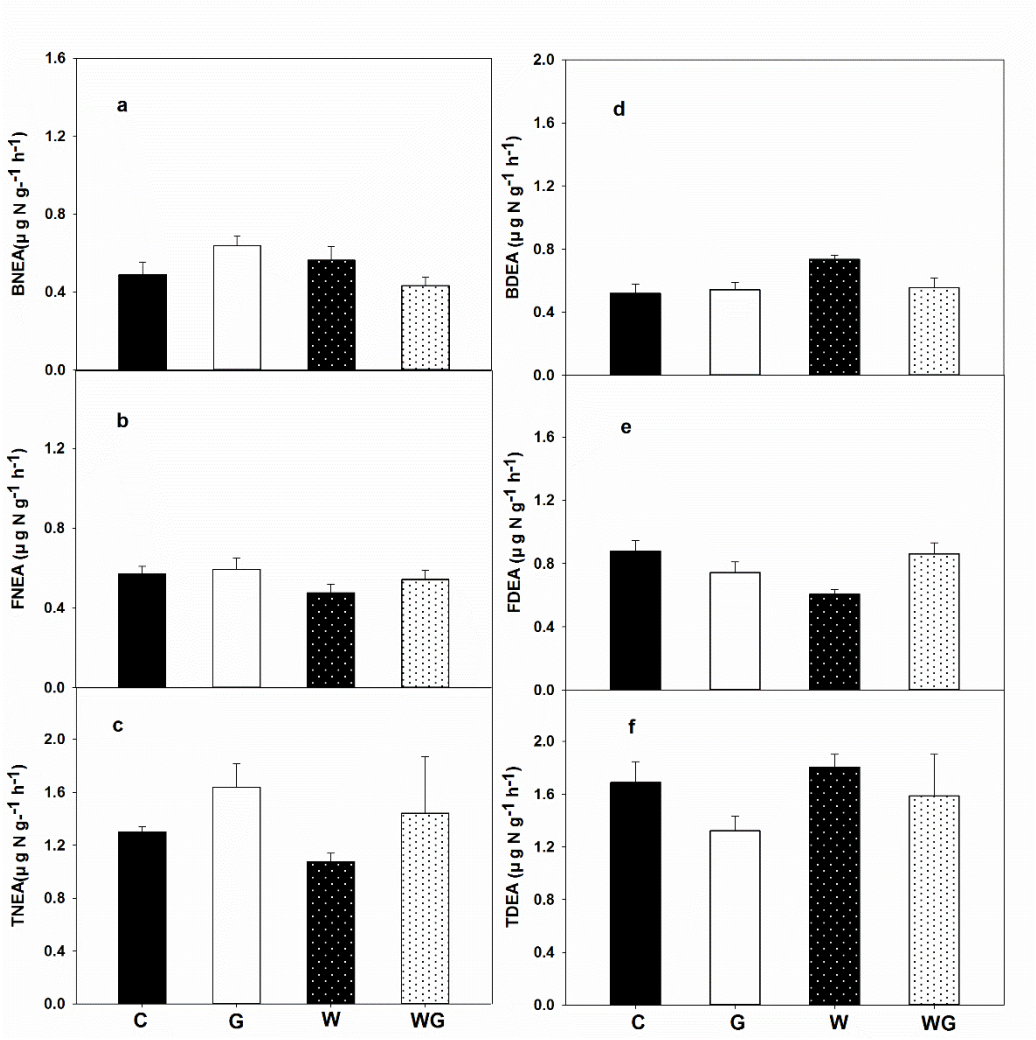
638

639

640 Fig.4

641

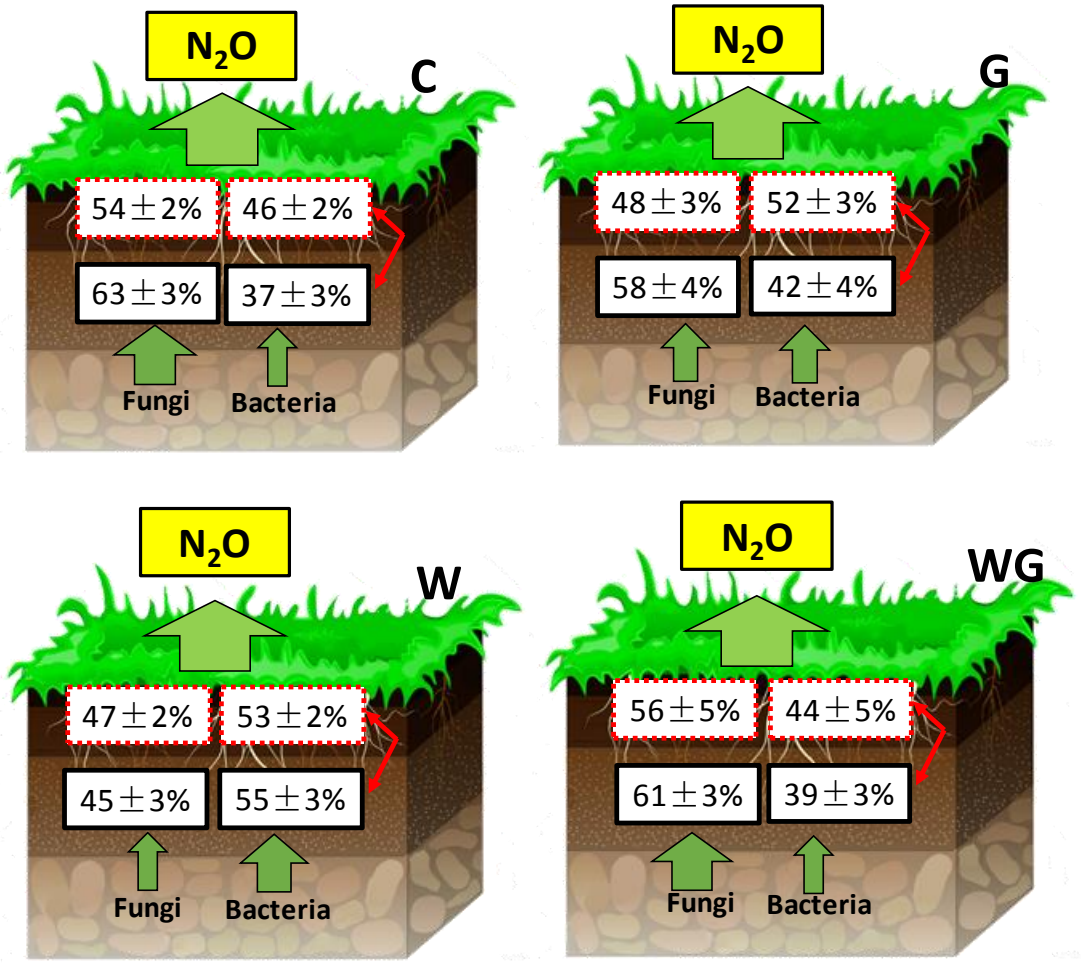
642



643

644 Fig.5

645



646