

1 **Fungi regulate response of N₂O production process to warming and**
2 **grazing in a Tibetan grassland**

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4 Lei Zhong¹, Shiping Wang², Xingliang Xu³, Yanfen Wang⁴, Yichao Rui⁵, Xiaoqi Zhou⁶,
5 Qinhua Shen⁷, Jinzhi Wang⁸, Lili Jiang², Caiyun Luo⁹, Tianbao Gu¹, Wenchao Ma¹,
6 Guanyi Chen^{1, 10}

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8

9 ¹School of Environmental Science and Engineering, Tianjin University / China-
10 Australia Centre for Sustainable Urban Development, Tianjin 300350, China

11 ²Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau
12 Research, Chinese Academy of Sciences, Beijing 100101, China

13 ³Key Laboratory of Ecosystem Network Observation and Modeling, Institute of
14 Geographic Sciences and Natural Resources, Chinese Academy of Sciences,
15 Beijing 100101, China

16 ⁴University of Chinese Academy of Sciences, Beijing 100049, China

17 ⁵ Department of Soil Science, University of Wisconsin-Madison, Madison, WI 53706,
18 USA

19 ⁶ Tiantong National Forest Ecosystem Observation and Research Station, Center for
20 Global Change and Ecological Forecasting, School of Ecological and Environmental
21 Sciences, East China Normal University, Shanghai 200241, China

22 ⁷ Institute of Agriculture and Environment, Massey University, Private Bag 11222,
23 Palmerston North 4442, New Zealand.

24 ⁸ Beijing Key Laboratory of Wetland Services and Restoration, Institute of Wetland
25 Research, Chinese Academy of Forestry, Beijing 100091, China

26 ⁹ Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of
27 Plateau Biology, Chinese Academy of Sciences, Xining 810008, China

28 ¹⁰School of Science, Tibet University, No. 36 Jianguo Street, Lhasa 850012, Tibet
29 Autonomous Region, China

30

31

32 Author for correspondence:

33 Dr. Wenchao Ma; Prof. Guanyi Chen

34 School of Environmental Science and Engineering, Tianjin University / China-
35 Australia Centre for Sustainable Urban Development, Tianjin 300072, China

36 Email: mawc916@tju.edu.cn; chenguan yi@utibet.edu.cn

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40 **Abstract**

41 Lack of understanding of the effects of warming and winter grazing on soil fungal
42 contribution to nitrous oxide (N₂O) production process has limited our ability to predict
43 N₂O fluxes under changes in climate and land use management, because soil fungi play
44 an important role in driving terrestrial N cycling. A controlled warming and winter
45 grazing experiment included control (C), winter grazing (G), warming (W) and
46 warming with winter grazing (WG) was conducted to investigate the effects of warming
47 and winter grazing on soil N₂O production potential in an alpine meadow on the Tibetan
48 Plateau. Our results showed that soil bacteria and fungi contributed $46 \pm 2\%$ and 54
49 $\pm 2\%$ to nitrification, and $37 \pm 3\%$ and $63 \pm 3\%$ to denitrification in control treatment,
50 respectively. We conclude that soil fungi could be the main source for N₂O production
51 potential for the Tibetan alpine grasslands. In our results, neither warming nor winter
52 grazing affected the activity of enzymes responsible for overall nitrification and
53 denitrification. However, warming significantly increased the enzyme activity of
54 bacterial nitrification and potential of N₂O production from denitrification to $53 \pm 2\%$
55 and $55 \pm 3\%$, respectively, but decreased them to $47 \pm 2\%$ and $45 \pm 3\%$, respectively.
56 Winter grazing had no such effects. Warming and winter grazing may not affect the soil
57 N₂O production potential, but climate warming can alter biotic pathways responsible
58 for N₂O production process. These findings confirm the importance of soil fungi in soil
59 N₂O production process and how its responses to environmental and land use changes
60 in alpine meadow ecosystems. Therefore, our results provide some new insights about
61 ecological controls on N₂O production process and contribute to the development of

62 ecosystem nitrogen cycle model.

63

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

65

66 1 Introduction

67 N₂O emissions from soil contribute to climate warming as N₂O is a potent
68 greenhouse gas (Change, 2015), it is mainly produced in soils through microbial
69 nitrification and denitrification (Zumft, 1997). Clarifying nitrification and
70 denitrification processes and their controlling factors will be beneficial for
71 understanding N cycle in terrestrial ecosystems. Previous studies are mainly focused on
72 bacterial nitrification and denitrification (Hayatsu et al., 2008; Klotz and Stein, 2008)
73 because the conventional N cycle is thought to be controlled primarily by bacteria.
74 However, recent studies using novel molecular techniques have shown that soil fungi
75 are important players in terrestrial N cycling, including N₂O production and
76 nitrification/denitrification in drylands or soils with high organic carbon (C) and N
77 (Chen et al., 2015; Huang et al., 2017; Laughlin and Stevens, 2002; Marusenko et al.,
78 2013; Zhong et al., 2018).

79 The Tibetan grasslands occupy approximately 40% of the Tibetan Plateau which
80 represents 0.7-1.0% of total global N storage (Tian et al., 2006) and is required for
81 sufficient forage production (Zheng et al., 2000). These grasslands represent one of the
82 most vulnerable regions in the world to climate change and anthropogenic perturbation
83 (Thompson et al., 1993; Thompson, 2000; Wang and French, 1994). A much greater than
84 average increase in the surface temperature has been predicted to occur in this region
85 in the future (Giorgi et al., 2001) and have profound impacts on soil N cycling in alpine
86 grasslands. Additionally, the grasslands of the Tibetan Plateau are generally divided
87 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 N₂O 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 N₂O 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 N₂O 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 N₂O
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

110 al., 2005), higher organic C/N (Chen et al., 2015) and a more arid soil environment
111 (Marusenko et al., 2013) compared to bacteria. Climate warming and grazing can
112 change vegetation cover, soil water and energy balance, alter the quantity and quality
113 of soil organic matter and mineral N content (Saggar et al., 2004), and thus affect N₂O
114 production (Shi et al., 2017). However, it remains unknown how bacteria and fungi
115 respond to concurrent warming and grazing and contribute to N₂O production in alpine
116 grasslands.

117 To clarify whether fungi played the mainly role in N₂O production process and its
118 response to warming and winter grazing in alpine grasslands, we used a warming and
119 grazing experiment over 10 years in an alpine meadow on the Tibetan Plateau. We
120 measured the gene abundance of soil bacterial and fungal communities using
121 quantitative PCR, and the potential of N₂O emission from bacterial and fungal
122 nitrification and denitrification through an incubation experiment to assess the
123 contribution of N₂O production potential from bacteria and fungi. We aimed to test the
124 following hypotheses: (1) soil fungi were the main contributor to N₂O production
125 because of the low soil temperature and high organic C and N in the alpine grasslands,
126 and (2) although N₂O emission was not affected by warming and winter grazing at our
127 site (Zhu et al., 2015), the biotic pathways responsible for N₂O would be changed due
128 to the distinct preferred soil environments of bacterial and fungal communities.

129

130 **2 Materials and Methods**

131 **2.1 Site and sampling.** Details of the experimental site and design of the warming and

132 grazing were described by Wang et al. (2012). The experiment was conducted in an
133 alpine grassland (37°37'N, 101°12'E, 3250 m elevation) at the Haibei Alpine Meadow
134 Ecosystem Research Station of the Chinese Academy of Sciences. Over the past 25
135 years, the mean annual temperature was -2 °C, and the mean annual precipitation was
136 500 mm. In soil sampling year and month of 2015, mean temperature was 0 °C and 9.7
137 °C, respectively; total rainfall was 327.2 mm and 46.6 mm, respectively. Over 80% of
138 total rainfall falls during the summer monsoon season (Luo et al., 2010; Zhao and Zhou,
139 1999). The soil was classified as Gelic Cambisols (WRB, 1998). The plant community
140 at the experimental site is dominated by *Kobresia humilis*, *Festuca ovina*, *Elymus*
141 *nutans*, *Poa pratensis*, *Carex scabrirostris*, *Gentiana straminea*, *Gentiana farreri*,
142 *Blysmus sinocompressus*, *Potentilla nivea* and *Dasiphora fruticosa* (Luo et al., 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 1.2°C during
155 the daytime and 1.7°C 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

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

180

181 **2.3 Soil properties and gene abundance of bacteria and fungi analysis.** Soil
182 moisture content was measured by drying at 105°C for 24 hours. For soil mineral N
183 (NH₄⁺-N and NO₃⁻-N) analyses, 10 g of soil (field-moist) was shaken for 1 hour with
184 50 mL of 1 M KCl and filtered through filter paper, and determine the NH₄⁺-N and NO₃⁻
185 -N concentrations by Skalar flow analyzer (Skalar Analytical, Breda, The Netherlands).
186 Total C and N content were measured by using combustion elemental analyzers
187 (PerkinElmer, EA2400, USA).

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

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

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

204 **2.4 Total, fungal and bacterial nitrification enzyme activity, and total, fungal and** 205 **bacterial potential of N₂O production from denitrification analysis.**

206 Fungal (FNEA), bacterial (BNEA) and total nitrification enzyme activity (TNEA)
207 were determined following the protocol described in Dassonville et al. (2011). Briefly,
208 moist field soil equivalent to 12 g of dry soil was weighed into 240-mL specimen bottles
209 (LabServ), 12 mL of NH₄-N solution (50 µg N-(NH₄)₂SO₄ g⁻¹ dry soil) and distilled
210 water was added to achieve a 96 mL total liquid volume, and the slurry was incubated
211 at 28°C for 10 hours with constant agitation (180 rpm) in an orbital shaker (Lab-Line
212 3527; Boston, MA, USA) to mix slurry well and provide an aerobic environment. Three
213 treatments were imposed: (I) cycloheximide (C₁₅H₂₃NO₄, a fungicide) at 1.5 mg g⁻¹ in
214 solution was used to inhibit the nitrification activity from soil fungi, (II) streptomycin
215 sulphate (C₄₂H₈₄N₁₄O₃₆S₃, a bactericide) at 3.0 mg g⁻¹ in solution was used to inhibit
216 the nitrification activity from soil bacteria (Castaldi and Smith, 1998; Laughlin et al.,
217 2009) and (III) a no-inhibitor control was used to show the total nitrification activity.

218 During incubation, 10 mL of the soil slurry was sampled with a syringe at 2, 4, 6, 8 and
219 10 h, and then filtered through Whatman No. 42 ashless filter paper. Filtered samples
220 were stored at -20 °C until analysis for $\text{NO}_2^- + \text{NO}_3^-$ concentration on a LACHAT
221 Quickchem Automated Ion Analyzer (Foss 5027 Sampler, TECATOR, Hillerød,
222 Denmark). Linear regression between the $\text{NO}_2^- + \text{NO}_3^-$ production rate and time was
223 observed, and the rates of nitrification enzyme activity were determined from the slope
224 of this linear regression. The nitrification enzyme activity of soil fungi was estimated
225 by the difference between rates of nitrification enzyme activity under treatment (III)
226 and treatment (I); the nitrification enzyme activity of soil bacteria was estimated by the
227 difference between rates of nitrification enzyme activity under treatment (III) and
228 treatment (II). The total nitrification enzyme activity was from treatment III.

229 Fungal (FDEA), bacterial (BDEA) and total potential of N_2O production (TDEA)
230 from denitrification was measured in fresh soil from each plot following the protocol
231 described in Patra et al. (2006) and Marusenko et al. (2013). Three sub-samples
232 (equivalent to 12 g dry soil) from each soil sample were placed into 240-mL plasma
233 flasks, and 7 mL of a solution containing KNO_3 ($50 \mu\text{g NO}_3^- \text{N g}^{-1}$ dry soil), glucose
234 (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) were added. Additional
235 distilled water was provided to achieve 100% water-holding capacity and optimal
236 conditions for denitrification. Three treatments were imposed: (I) cycloheximide
237 ($\text{C}_{15}\text{H}_{23}\text{NO}_4$; a fungicide) at 1.5 mg g^{-1} in solution was used to inhibit the fungal
238 potential of N_2O production from denitrification, (II) streptomycin sulphate
239 ($\text{C}_{42}\text{H}_{84}\text{N}_{14}\text{O}_{36}\text{S}_3$; a bactericide) at 3.0 mg g^{-1} in solution was used to inhibit the bacterial

240 potential of N₂O production from denitrification (Castaldi and Smith, 1998; Laughlin
241 and Stevens, 2002), and (III) a no-inhibitor control was used to show the total potential
242 of N₂O production from denitrification. The headspace air of the specimen bottles was
243 replaced with N₂ to provide anaerobic conditions. Specimen bottles were then sealed
244 with a lid containing a rubber septum for gas sample collection. Specimen bottles with
245 the soil slurry were then incubated at 28°C for 48 h with constant agitation (180 rpm)
246 in an orbital shaker (Lab-Line 3527; Boston, MA, USA). During incubation, 12-mL gas
247 samples was taken at 0, 24 and 48 h with syringes and injected into pre-evacuated 6-
248 mL glass vials. The N₂O concentration of the gas samples was analyzed via gas
249 chromatography. The potential of N₂O production from denitrification were calculated
250 from the slope of the regression using values for 0, 24 and 48 hours of incubation. The
251 fungal potential of N₂O production from denitrification was estimated by the difference
252 between potential production under treatment (III) and treatment (I); The bacterial
253 potential of N₂O production from denitrification was estimated by the difference
254 between rates of denitrification enzyme activity under treatment (III) and treatment (II).
255 Total denitrification enzyme activity was from Treatment III.

256 For the contribution of bacteria and fungi to total nitrification enzyme activity was
257 calculated it by the ratio of BNEA or FNEA to BNEA+FNEA; the contribution of
258 bacteria and fungi to total potential of N₂O production from denitrification was
259 calculated it by the ratio of BDEA or FDEA to BDEA+FDEA.

260 **2.5 Statistical analysis.** For the controlled experiment, the statistical significance of

261 the effects of warming, grazing and their interaction on plant biomass, soil properties,
262 microbial functional genes, and fungal and bacterial nitrification enzyme activity and
263 potential of N₂O production from denitrification were tested by two-way ANOVA in
264 the PROC GLM procedure of SAS (version 9, SAS Institute, Cary, NC, USA).

265

266 **3 Results**

267 **3.1 Plant biomass and soil properties**

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

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

276 Soil total C (TC) was not affected by grazing (P=0.13) or warming (P=0.12) alone,
277 but there was a marginal interaction between grazing and warming on TC (P=0.07) (Fig.
278 2a, Table 1). Similar to TC, soil total N (TN) also showed no response to grazing or
279 warming (Fig. 2b, Table 1). Soil NH₄⁺-N content was lower in warming treatments than
280 in no-warming treatments (P=0.05) (Fig. 2c, Table 1). Greater soil NO₃-N content
281 occurred under the warming treatments (P=0.05) than under the no-warming treatments
282 (Fig. 2d, Table 1).

283

284 **3.2 Microbial functional genes**

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

290 **3.3 Nitrification enzyme activity and potential of N_2O production from** 291 **denitrification of bacteria and fungi**

292 TNEA varied from 1.07 to 1.64 $\mu\text{g N g}^{-1} \text{h}^{-1}$ in all treatments. BNEA ranged from
293 0.43 to 0.64 $\mu\text{g N g}^{-1} \text{h}^{-1}$, which was lower than the FNEA in soil (0.59–0.66 $\mu\text{g N g}^{-1}$
294 h^{-1}) ($P = 0.01$) (Fig. 4 a-c). FNEA was lower under warming treatments than under the
295 no-warming treatments ($P = 0.05$) (Table 1).

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

300 **3.4 The contribution of bacteria and fungi to potential N_2O emissions**

301 The contribution of FNEA to TNEA varied from $47 \pm 2\%$ to $56 \pm 5\%$, and the
302 contribution of FDEA to TDEA varied from $45 \pm 3\%$ to $63 \pm 3\%$ (Fig. 5). Warming

303 significantly decreased the contribution of FNEA and FDEA to TNEA and TDEA in
304 soils (FNEA: P=0.02; FDEA: P=0.04).

305

306 **4 Discussion**

307 N₂O 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 N₂O 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 N₂O 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 °C; even during the
322 sampling day the mean temperature was only 11 °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 N₂O 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 N₂O 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 N₂O production potential on the Qinghai-Tibetan Plateau, the biotic pathways
342 responsible for N₂O had been changed, as bacterial contribution to TNEA and TDEA
343 all were higher than fungal which suggested the higher bacterial N₂O production
344 potential under warming treatment (Fig. 4, Table 1). The increase in bacterial N₂O
345 production potential, coupled with a decrease in fungal N₂O production potential, could
346 be the main reasons why the total N₂O production potential was no difference between
347 control and warming treatments. The field data of N₂O emission in our site was

348 measured in the year of 2011–2012 also showed no effect of warming on N₂O 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 N₂O
351 to warming.

352 It is the two reasons that lead to the changes of fungal and bacterial pathways for
353 N₂O 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 NH₄⁺-N concentration did not change with warming,
359 soil NO₃⁻-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 N₂O production did
376 not change, with a shift in dominance from fungi to bacteria in N₂O 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 N₂O
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 trampling (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 N₂O
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

392 environmental conditions for both winter grazing and control had no effect on soil fungi
393 and bacteria, and thus on fungal and bacterial nitrification and denitrification. Moreover,
394 the field data of N₂O emission in the year of 2011-2012 also support the results and
395 suggest that replacing summer grazing by winter grazing could cause the soil N cycle
396 process to become stable (Zhu et al. 2015).

397 Overall, we conclude that fungi played the dominant role in the N₂O production
398 process in alpine meadows. Previous study had proved the climate warming did not
399 affect the N₂O production in our site (Zhu et al. 2015), but we found warming could
400 alter biotic pathways responsible for N₂O production process on the Tibetan Plateau.
401 Our study exhibited the effects of a decade of the 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 a 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 N₂O 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

413

414 **Competing interests**

415 The authors declare that they have no conflict of interest.

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583

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 \pm 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 \pm 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 \pm 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 \pm 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 \pm 1 s.e.m. ($n=4$).

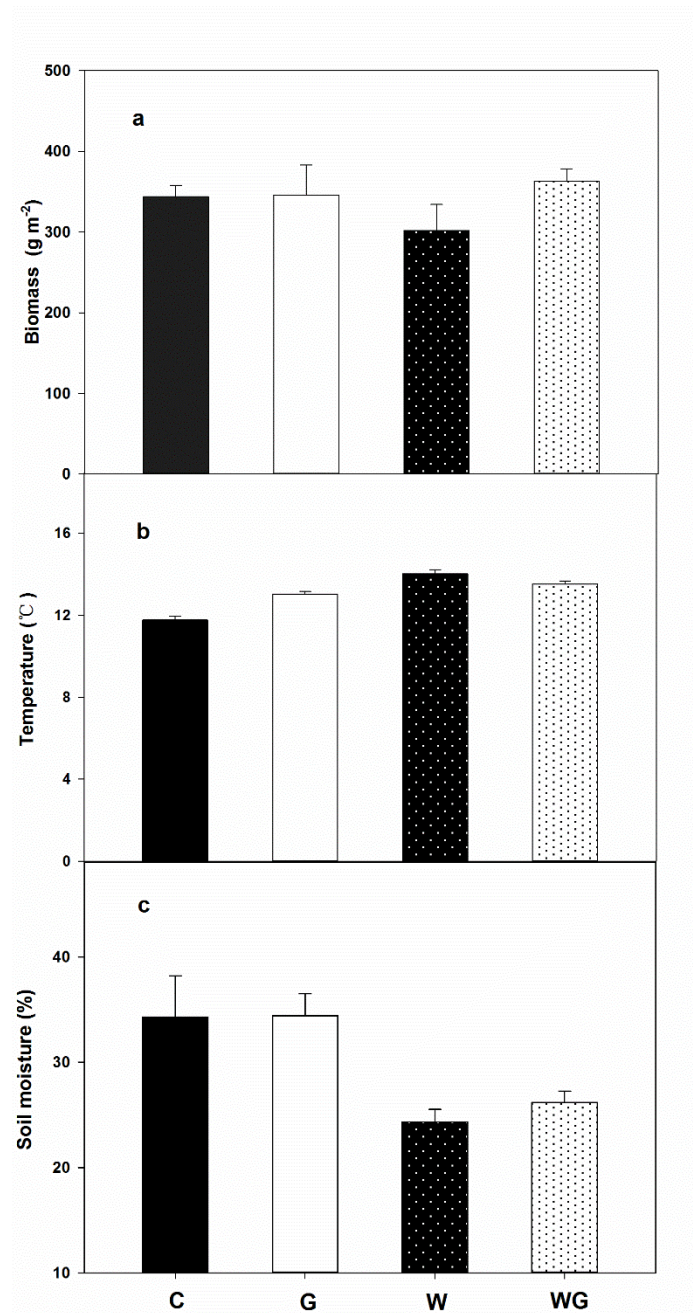
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625 Fig.1



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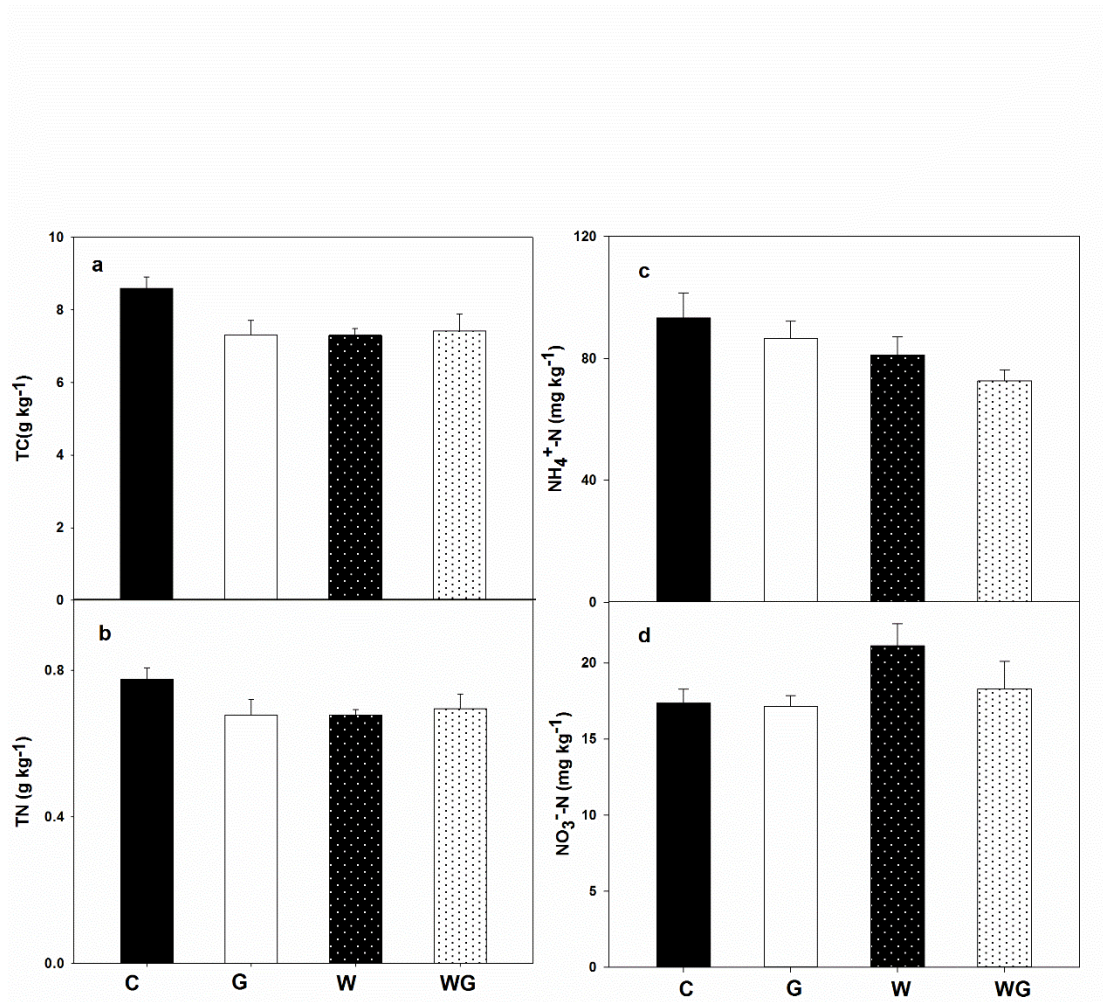
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628 Fig. 2

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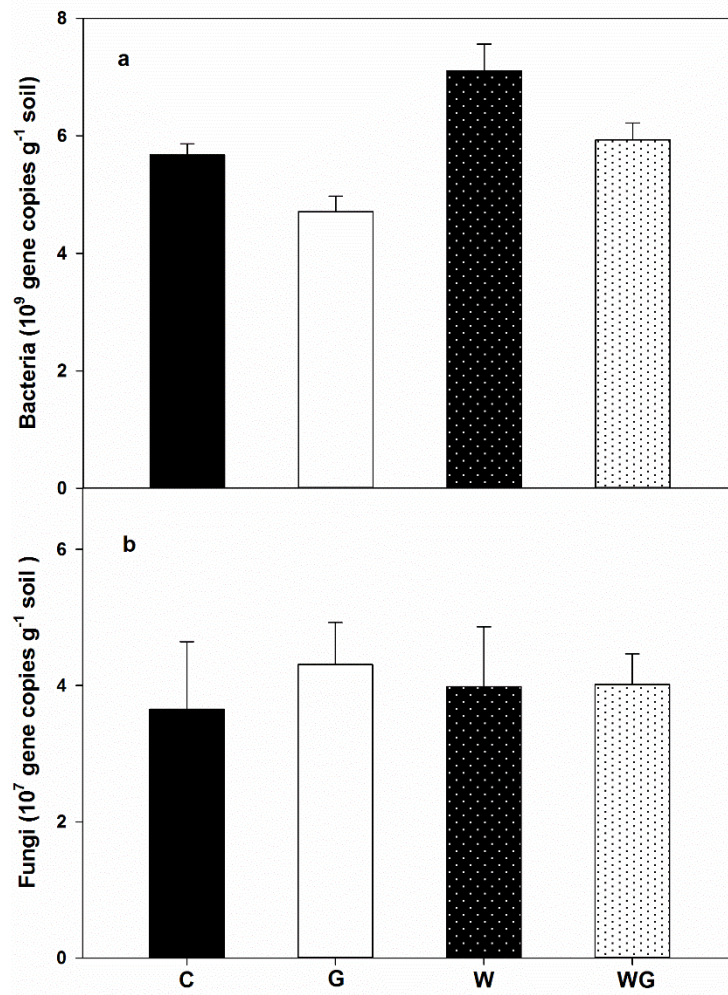
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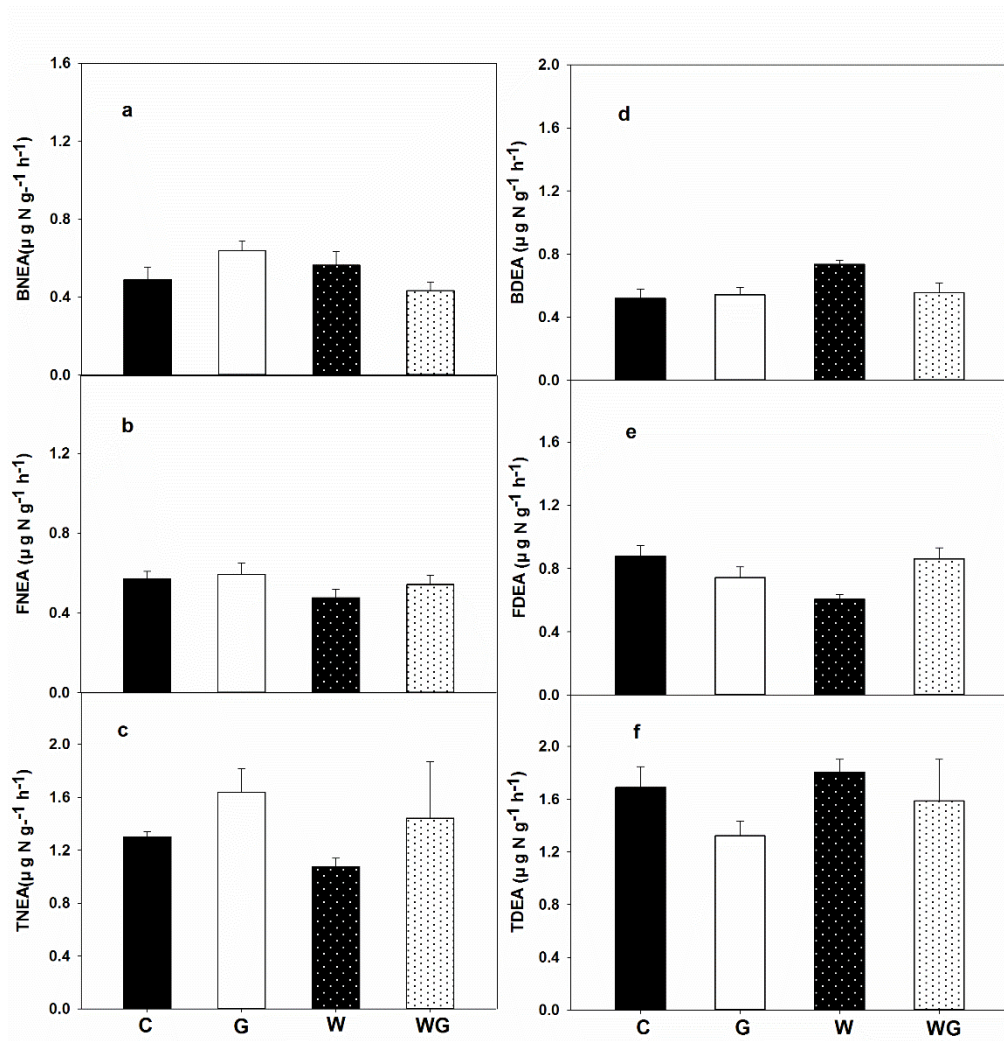
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633 Fig. 3
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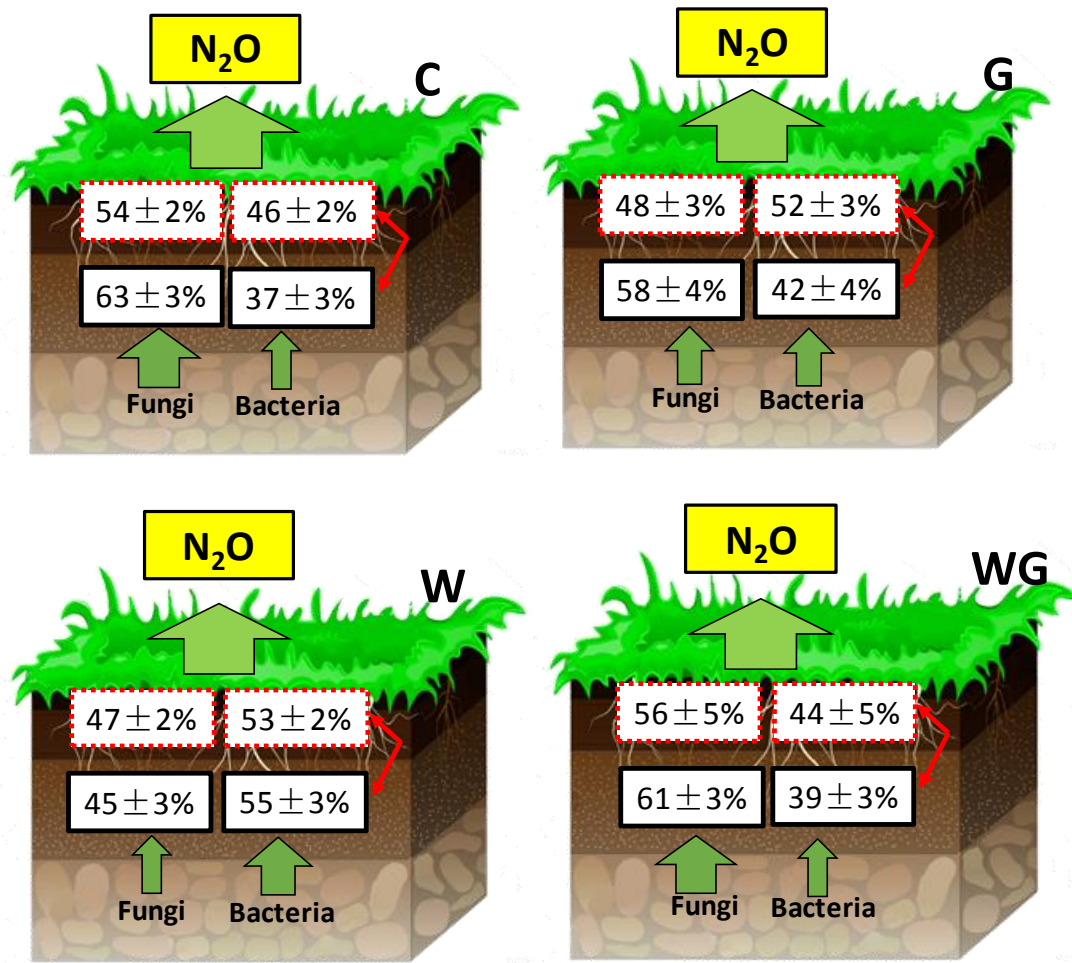
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640 Fig.4
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