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Warming counteracts grazing effects on the functional structure of the soil microbial community in a Tibetan grassland

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ABSTRACT

Grazing intensity and global warming are expected to increase in the forthcoming decades, with uncertain consequences for their interaction on grassland ecosystems and their functions. We investigated the effects of warming, grazing and their interaction in a factorial warming (+1.2–1.7 °C) and grazing (moderate intensity with ca. 50% vegetation consumption) experiment in a Tibetan alpine meadow on microbial communities by studying functional genes involved in soil carbon and nitrogen cycles, using GeoChip technology. Our results showed that microbial functional gene structure and abundances were largely affected by the interactive effect of grazing and warming, rather than the main effect of warming or grazing. Compared to the control, grazing alone significantly increased the functional gene alpha diversity, changed the overall functional community structure, and increased the abundances of C fixation, C degradation, N mineralization and denitrification genes, likely due to the stimulating impact of urine and dung deposition. Warming alone did not change these microbial properties, possibly related to the unchanged soil nutrient status. Despite an increase in soil NO_3^- concentrations and the deposition of urine and dung, the combined treatment did not change functional gene alpha diversity, community structure, or C/N cycling gene abundances, possibly resulting from the limiting effect of water depletion in the combined treatment. Our study revealed antagonistic interactions between warming and grazing on microbial functional gene structure and abundances, which remained stable under the moderate intensity of grazing in future warming scenario in the Tibetan alpine meadow, raising potentially important implications for predicting future soil carbon and nitrogen processes in these systems.

1. Introduction

Grassland ecosystems, accounting for over 40% of the terrestrial surface, are faced with remarkable sustainability challenges, including those induced by livestock grazing and climate warming (Lu et al., 2013; Zhou et al., 2017). Livestock grazing is a dominant land use activity for some grassland ecosystems (Chen et al., 2013), and important for ecosystem services, e.g. biodiversity conservation and primary

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production (McSherry and Ritchie, 2013; Yan et al., 2013). Overgrazing is recognized as one of the two main causes for grassland degradation (Harris, 2010), along with global climate change (Liu et al., 2018). Global mean air temperature had increased by 0.85 °C from 1850 to 2012 and is predicted to increase by at least 2 °C by the end of this century (2081–2100) compared to the period of 1850–1900 (IPCC, 2013). Thus, it might be inevitable that grazing and climate warming would occur simultaneously in grassland ecosystems in future scenarios (Li et al., 2018).

Grazing has substantial influences on grassland ecosystems as it inevitably involves deposition of urine and dung, generally decreases soil porosity through trampling, and typically changes litter quality and root exudates via defoliation (Patra et al., 2006; Zhong et al., 2018). According to a global meta-analysis, the grazing effect on soil carbon (C) and nitrogen (N) pools depends on its intensity in grassland ecosystems: low grazing intensity tends to increase soil C and N pools, while high grazing intensity has opposite effects (Yan et al., 2013; Zhou et al., 2017), though definitions of “low” and “high” intensities are not uniform in the literature. With respect to microbial functional genes involved in N cycling, which have important implications for soil biogeochemical processes, free grazing was found to increase abundances of N mineralization and nitrification genes, but decrease denitrification gene abundance in an alpine meadow (Yang et al., 2013). In two semi-arid grasslands, significantly decreased ammonia-oxidizing and denitrifying genes were observed under moderate grazing (Ding et al., 2015). In relation to microbial functional genes involved in C cycling, they have been found to be significantly reduced by severe grazing in an alpine meadow, including those involved in C degradation and fixation, and CH_4 cycling (Yang et al., 2013; Wang et al., 2016).

Climate warming has been found to regulate soil C and N pools in grassland ecosystems by changing soil C and N inputs as well as increasing litter decomposition and soil respiration (Lu et al., 2013). It was reported that warming could affect the quality and quantity of soil C and N inputs via increasing plant biomass and shifting plant community composition (Bardgett et al., 2008; Classen et al., 2015), e.g. increasing non-leguminous forb biomass (Lin et al., 2010) and the ratio of C_4 to C_3 grass biomass (Epstein et al., 2002; Morgan et al., 2011). Soil microbial communities could be affected either by these soil substrate changes mediated by plant communities or the direct warming impacts through soil temperature and moisture alterations (Bardgett et al., 2008; Classen et al., 2015). Changes in microbial functional genes were observed under warming (Trivedi et al., 2016; Xue et al., 2016b; Cheng et al., 2017). For example, warming significantly increased abundances of *amoA* and *nosZ* and changed community structure of *nirK* lineages in an upland grassland, which were closely related to N_2O emissions (Cantarel et al., 2012). In addition, stimulation of genes for decomposing labile C but not recalcitrant C was observed as a crucial microbially-mediated mechanism for maintaining soil C stability under warming in a tall-grass prairie ecosystem (Zhou et al., 2011).

Strong interactions between grazing and warming have been observed for important ecosystem processes and properties (Li et al., 2018). For example, though grazing amplified responses of air and soil temperatures to warming (Klein et al., 2005), it mitigated the warming impacts on plant community productivity and composition through defoliation in an alpine grassland ecosystem (Klein et al., 2007; Post and Pedersen, 2008). On the other hand, warming offset grazing effects on plant communities through increasing plant height, aboveground biomass and vegetation living state, but decreasing plant diversity in alpine grasslands (Zhang et al., 2015). However, little information is available on interactive effects of warming and grazing on functional structure of soil microbial community in grasslands (Li et al., 2016). Functional structure of soil microbial community is critical for assessing ecosystem functioning changes given their key roles in driving soil biogeochemical processes, especially those involved in soil C and N cycling (He et al., 2010; Zhou et al., 2011).

The Tibetan Plateau is the largest geo-morphological unit in the Eurasian continent and is among the most sensitive eco-regions to climate changes and anthropogenic disturbances (Wang et al., 2009; IPCC, 2013). Air temperature increase in the Tibetan Plateau is significantly faster than the global average (Wang et al., 2008). Alpine grasslands of Tibetan Plateau cover an area of about 2.5 million km^2, with 40% of land being alpine meadows (Cao et al., 2004). Alpine meadows contribute considerably to global soil C and N pools and almost all alpine meadows are used for grazing in Tibetan Plateau (Wen et al., 2013; Li et al., 2014; Ding et al., 2016). At the Haibei Alpine Meadow Ecosystem Research Station in northeastern Qinghai-Tibetan Plateau, an experimental facility was established in 2006 to study the impacts of grazing and climate warming on the alpine meadow ecosystems. The facility includes controlled asymmetrical warming (warmed 1.2 °C at daytime; 1.7 °C at nighttime) and grazing (moderate intensity with ca. 50% vegetation consumption) at the field scale (Kimball et al., 2008; Luo et al., 2010). In this field, previous studies have found that warming offset the negative effects of grazing on plant communities through increasing aboveground net primary productivity (Wang et al., 2012), enlarging labile C/N pools (Rui et al., 2011), and accelerating litter decomposition (Luo et al., 2010), though below microbial mechanisms are still unclear. Here, we focus on grazing, warming and their interactive effects on soil microbial functional genes in the alpine meadow to test hypotheses: (1) warming enhances soil substrates through increasing plant growth, and thus stimulates corresponding function genes involved in C degradation and N cycling from soil microbial community; (2) grazing decreases soil substrates through animal grazing behaviour, and thus inhibits corresponding function genes involved in C degradation and N cycling from soil microbial community; (3) warming counteracts effects of grazing on microbial functional genes involved in C degradation and N cycling.

2. Materials and methods

2.1. Site and sampling

The experiment was carried out at the Free-Air Temperature Enhancement (FATE) facility at the Haibei Alpine Meadow Ecosystem Research Station (37°37′ N, 101°12′ E, and 3250 m elevation) in northeastern Qinghai-Tibetan Plateau. The mean annual temperature is -2 °C and mean annual precipitation is 500 mm (Zhao and Zhou, 1999; Luo et al., 2010). Over 80% of precipitation falls during the summer monsoon season. The soil at the study site is classified as Mat Crygelic Cambisols with a texture of clay loam. The total C, total N, bulk density and porosity of soils were 73.3 g kg^{-1}, 5.5 g kg^{-1}, 1.09 g cm^{-3} and 58.7%, respectively (Cao et al., 2008; Lin et al., 2015). The plant community in the experimental site is dominated by *Kobresia humilis* (C.A.Mey. ex Trautv.) Serg., *Festuc aovina* L., *Elymus nutans* Griseb., *Poa pratensis* L., *Care xscabrirostris* Kük., *Scripus distigmaticus* (Kukenth.) Tang et Wang, *Gentiana straminea* Maxim., *Gentiana farreri* Balf.f., *Blysmus sinocompressus* Tang & F.T.Wang and *Potentilla nivea* L. (Luo et al., 2010).

Using infrared heating to mimic climate warming, FATE increased plant canopy temperature by 1.2 °C during daytime and 1.7 °C at night in summer (Kimball et al., 2008; Luo et al., 2010). Such warming manipulations were intended to mimic the expected temperature changes by the year of 2075 in this area. A two-factorial design, consisting of control (C), warming alone (W), grazing alone (G), and the combined treatment of warming and grazing (WG), has been adopted to investigate effects of simulated warming and grazing on alpine meadow ecosystem since May 2006. A total of 16 circular plots with 3 m diameter were arranged using a completely randomized design with four replicates for each treatment. The circular plots were 3 m apart between neighbouring plots. The livestock grazing was initially implemented by fencing one adult Tibet sheep in the grazing plots for nearly 2 h on 15th August 2006, and two adult Tibetan sheep were fenced for approximately 1 h in the grazed plots on the mornings of 12th July, 3rd August

and 12th September 2007, 8th July and 20th August in 2008, 9th July in 2009. All experimental sheep were fenced into three additional 5*5 m fenced plots for one day before every grazing treatment to help them to adapt to smaller plots on the following day. The canopy heights after grazing were about half of the initial height and annual average vegetation utilization rate was 48.5% in G and 47.9% in WG, which corresponded to a moderate grazing intensity (Cao et al., 2004).

The soil temperature at depths of 5, 10 and 20 cm was measured automatically using type K thermocouples (Campbell Scientific, Logan, Utah, USA), and values for 1 min and 15 min averages were stored. Soil moisture at depths of 10, 20, 30 and 40 cm was manually measured through a tube in the ground down to 40 cm depth using a frequency domain reflectometer (FDR; Model Diviner-2000, Sentek Pty Ltd., Australia) at 08:00, 14:00, and 20:00 every day (Luo et al., 2010; Wang et al., 2012).

Five soil cores (5 cm diameter) were randomly collected within each plot on 3rd August 2009 at the depth of 0–20 cm and then mixed as a composite sample. After transporting to the laboratory, all soil samples were sieved through a 2 mm mesh, removed visible roots by hand, and stored at −2 °C for molecular analysis and 4 °C for soil chemical analyses.

2.2. Measurement of soil and plant properties

The soil moisture, pH, NH_4^+-N, NO_3^--N, microbial biomass C and N (MBC and MBN), soluble organic C and N (SOC and SON), total N (TN) and phosphorus (TP) have been measured and reported by Rui et al. (2011), in which soils were collected in the same sampling campaign in 2009 though seprated into 0–10 cm and 10–20 cm depths. Aboveground plant biomass (Biomass*A*), belowground root biomass (Biomass*B*) and plant species diversity were measured and reported by Wang et al. (2012), which were also used for the plant analyses in this study.

2.3. DNA extraction and purification

Genomic DNA was extracted from 0.5 g subsample using the FastDNA Spin kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. The 30 μlextracted DNA was further purified as described by Yang et al. (2013). The quality of purified DNA was assessed by ratios of $A_{260/280}$ and $A_{260/230}$ using a ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and total microbial DNA concentrations were measured by using Qubit quantification platform with Quant-iT™ dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA).

2.4. GeoChip analysis

GeoChip analysis was performed according to the procedure described previously by Yang et al. (2013). Briefly, the purified DNA was labelled with fluorescent dye Cy5 using random primers. The labelled DNA was purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), and further dried in a SpeedVac (DNA Speedvac, Model DNA 100, Savant) at 45 °C for 45 min. The dried DNA was suspended in hybridization buffer. Hybridization reaction was conducted on a MAUI Hybridization Station (BioMicro Systems, Salt Lake City, UT, USA) at 42 °C for 12 h. After hybridization, microarrays were scanned by a Scan Array Express microarray scanner (PerkinElmer, Boston, MA, USA) using a laser power of 90% and a photomultiplier tube gain of 75%. The signal intensity of every spot was measured through analysing images with ImaGene 6.0 (Biodiscovery, EI Segundo, CA, USA). Spots flagged by ImaGene or having signal-to-noise ratio (SNR = signal mean-background mean/background standard deviation) < 2 were removed because of their poor quality. A gene was recognized as positive if it was detected in at least two of four replicates. Signal intensities of all the genes were normalized by a relative abundance method and natural logarithmic transformation subsequently.

2.5. Statistical analyses

Two-way analysis of variance (ANOVA) was used to examine main and interactive effects of warming and grazing on the gene diversity and relative abundances. Differences among treatments were performed by Turkey-adjusted least-square means for multiple comparisons (Russell, 2016). The directions of the significant interactions were classified as antagonistic or synergistic based on individual effects and 95% confidence intervals of effect sizes for interaction calculated by Hedges' *d* (Gurevitch et al., 2000; Crain et al., 2008). Permutation Multivariate Analysis of Variance (Adonis) based on Bray-Curtis distance matrices was used to test treatment effects on multivariate functional community compositions with 999 times permutation, in which the statistical significance was judged based on F-tests. Non-metric multidimensional scaling (NMDS) was performed to visualize the multivariate changes in overall functional community composition using Bray-Curtis distance. Redundancy analysis (RDA), a constrained ordination technique, was performed to explore the relationship between microbial community and biotic and abiotic exploratory variables. The predictors were selected by using manual forward selection with 999 Monte Carlo permutation ($P < 0.05$) and variance inflation (VIF < 25).

RDA was performed using Canoco 4.5 for Windows (Biometris, Wageningen, Netherlands) as suggested by Lepš and Šmilauer (2003). Alpha diversity indices of microbial functional communities, including richness, Shannon index (H) and Simpson index (1/D) (Valentini et al., 2009; Haegeman et al., 2013), NMDS and Adonis analysis were performed using R statistical program (R Development Core Team) running packages including vegan and lsmeans. All significant differences were judged by $P < 0.05$, unless otherwise stated.

3. Results

3.1. Effects of warming and grazing on overall soil microbial functional diversity

A total of 3,296 detected genes distributed in 224 functional gene families were derived by GeoChip 3.0 and used to assess soil microbial functional diversity. Alpha diversity indices including richness, Shannon index (H) and inverse Simpson index (1/D) were not affected by the main effect of warming or grazing, but there were significantly antagonistic interactions between warming and grazing for all these variables (Fig. 1). Compared to the control, grazing alone without warming had significantly higher gene richness and inverse Simpson index (1/D), which were not changed when grazing was combined with warming. Warming alone did not differ from the control on these diversity indices. The evenness of functional community was not significantly affected by the main effects of warming, grazing and their interaction, or any treatments (data not shown).

3.2. Effects of warming and grazing on microbial functional community structure

Pairwise comparisons for the overlapped genes showed that the control shared 62.3%–68.3% functional genes with grazing alone and warming alone treatments. NMDS analysis based on functional gene compositions in Fig. 2 illustrates that samples in grazing alone were clearly separated from those in the control, while samples in warming alone or combined with grazing treatment could not be separated from control samples completely.

Consistently, as shown in Table 1, pairwise comparisons of community structure dissimilarity by the Adonis test based on Bray-Curtis revealed that there was a significant ($P = 0.046$) difference between grazing alone and control, but not between warming alone and control

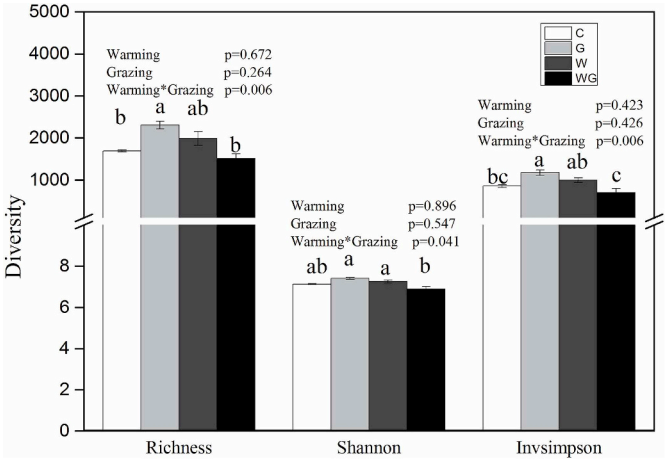

Fig. 1. Functional gene diversity indices under treatments of grazing alone (G), warming alone (W), warming combined with grazing (WG), and the control (C). Error bars represented standard errors (n = 4). P values for main and interactive effects were based on Two-way ANOVA for each diversity index. Different letters above the bars indicated significant differences among treatments ($P < 0.05$), determined by *post-hoc* lsmeans test following Two-way ANOVA.

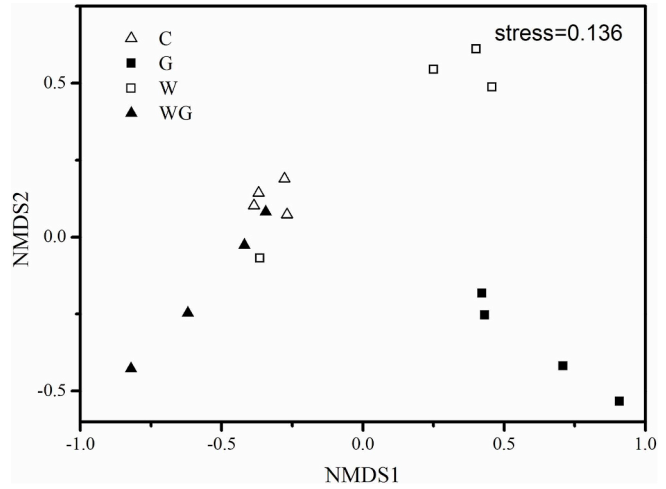

Fig. 2. Nonmetric Multidimensional Scaling (NMDS) profile for functional community composition of soil microorganisms based on GeoChip analysis and Bray-Curtis dissimilarity index. Only samples of grazing alone treatment (G) separated clearly from control (C), while samples of other treatments (W = warming along, WG = warming combined with grazing) did not separate completely from control.

Table 1
Main effects of warming, grazing and their interaction on functional gene structure of soil microbial communities by two-way permutational multivariate analysis of variance (Adonis) and pairwise comparisons between treatments by one-way Adonis test, based on Bray-Curtis index. **P < 0.05, *P < 0.1

		R^2	F-value	P-value
Main and interactive effects	Warming	0.067	0.987	0.454
	Grazing	0.066	0.989	0.386
	Warming × Grazing	0.146	0.178	0.004**
Pairwise comparison	C vs. G	0.252	2.496	0.046**
	C vs. W	0.265	2.393	0.058*
	C vs. WG	0.153	0.867	0.676
	W vs. WG	0.194	1.742	0.124
	G vs. WG	0.214	1.862	0.091*
	W vs. G	0.211	0.241	0.122

($P = 0.058$) or between the combined treatment and control ($P = 0.676$). Adonis results also showed that there was a significant ($P = 0.004$) interaction between warming and grazing on overall functional community structure (Table 1), while no significant main effect of warming or grazing was detected.

3.3. Functional genes associated with C cycling

Among 21 detected C degradation genes, the main effect of warming negatively affected only 3 of them significantly, i.e. *nplT* encoding neopullulanase for starch decomposition, the gene encoding manganese for hemicellulose decomposition and *glx* encoding glyoxal oxidase for lignin decomposition (Table 2); while none was significantly affected by the main effect of grazing (Table 2). Significantly antagonistic interactions between warming and grazing were observed for *amyA* encodingalpha-Amylase and *pulA* encoding pullulanase for starch decomposition; genes encoding *mannanase* and xylanase for hemicellulose decomposition; *CDH* encoding cellobiose dehydrogenase for cellulose degradation; the gene encoding *acetylglucosaminidase* for chitin decomposition; *glx* encoding glyoxal oxidase and *mnp* encoding manganese peroxidase for lignin decomposition (Table 2). Significantly synergistic interaction was only observed for *nplT* encoding neopullulanase II for starch decomposition (Table 2).

Grazing alone led to significantly higher abundances of C degradation genes in comparison with the control, including *amyA* encodingα-amylase, the gene encoding glucoamylase, *nplT* encoding neopullulanase II for starch degradation; the gene encoding mannanase, *xylA* encoding xylose isomerase for hemicelluloses degradation; *CDH* encoding cellobiose dehydrogenase for cellulose degradation; genes encoding *acetylglucosaminidase* and endochitinase for chitin degradation; and *glx* encoding glyoxal oxidase for lignin degradation (Fig. 3b). On the contrary, none of the detected C degradation genes were significantly changed by warming alone or combined with grazing treatment compared to the control.

With respect to C fixation genes, the abundance of *PCC* gene encoding propionyl-CoA carboxylase was significantly affected by the antagonistic interaction between warming and grazing, and decreased by the main effect of warming, but not by the main effect of grazing (Table 2). Grazing alone significantly increased the relative abundance of *PCC*, which was unchanged under warming alone or combined with grazing (Fig. 3a). Other C fixation genes were not significantly affected by the main effect of warming, grazing and their interaction, or any treatments.

Two genes involved in methane metabolism were detected in this study, including *mcrA* encoding methyl-coenzyme M reductase for methane production, and *pmoA* encoding methane monooxygenases for methane consumption. The abundances of *pmoA* and *mcrA* were not significantly affected by the main effects of warming, grazing and their interaction, or any treatments (Fig. 3c).

3.4. Key functional genes involved in N cycling

The main effect of warming significantly decreased *nosZ* for nitrous oxide reduction (Table 2) and *amoA* for ammonia-oxidizing ($P = 0.031$), while the main effect of grazing significantly increased *nosZ* (Table 2). Interactions between warming and grazing were significantly antagonistic for genes involved in assimilatory N reduction (*napA* and *NirB*) and denitrification (*nirK*, *norB* and *nosZ*) (Table 2). Grazing alone significantly increased abundances of genes for N-fixation (*nifH*), ammonification (*ureC*), denitrification (*narG*, *NirK*, *nirS*, *norB*, *nosZ*), assimilatory N reduction to ammonium (*NirB*) and dissimilatory N reduction (*napA*) by 30.8–103.0% compared to the control (Fig. 4). By contrast, warming alone or combined with grazing only significantly decreased abundances of *amoA*, but did not affect any other N cycling genes (Fig. 4).

Table 2
Main effects and individual effect sizes of grazing and warming, as well as their interaction effect sizes with 95% confidence intervals on genes that were significantly affected by interactive effects between grazing and warming based on two-way ANOVA. Significance of main effects of grazing or warming were based on two-way ANOVA and boldface represented $p < 0.05$. Individual effect sizes of warming and grazing, as well as their interaction, were calculated by using *Hedges' d*. Interaction types were labelled as synergistic or antagonistic, based on below criteria: for situations where individual effect sizes of warming and grazing were both negative or had opposite directions, the interactions were synergistic when their effect sizes < 0 or antagonistic when > 0. In cases where the individual effect sizes of warming and grazing were both positive, interactions were characterized as synergistic and antagonistic when their effect sizes > 0 and < 0, respectively.

	Response variable	Main effects of grazing (p value)	Main effects of warming (p value)	Individual effect sizes of Grazing	Individual effect sizes of Warming	Grazing and Warming interaction effect sizes	Lower 95% C.L.	Upper 95% C.L.	Interaction types
Diversity indices	Richness	0.267	0.673	2.11	1.39	−3.41	−5.70	−1.13	antagonistic
	Shannon index (H)	0.538	0.897	1.43	1.08	−2.45	−4.59	−0.32	antagonistic
	Invsimpson index (1/D)	0.520	0.463	2.08	1.29	−3.63	−5.96	−1.30	antagonistic
Signal intensity of C cycling genes	PCC	0.215	**0.013**	2.00	0.12	−1.41	−2.46	−0.36	antagonistic
	amyA	0.246	0.129	1.69	0.45	−1.17	−2.21	−0.13	antagonistic
	nplT	0.358	**0.012**	1.74	−0.03	−1.30	−2.31	−0.30	synergistic
	pulA	0.714	0.446	0.83	0.64	−0.99	−2.03	−0.04	antagonistic
	mannanase	0.308	**0.013**	2.24	0.46	−1.76	−2.83	−0.70	antagonistic
	xylA	0.877	0.066	1.04	0.07	−0.97	−2.00	0.07	–
	xylanase	0.13	0.102	2.68	1.16	−1.95	−3.03	−0.87	antagonistic
	CDH	0.532	0.678	1.81	1.33	−1.52	−2.58	−0.46	antagonistic
	acetylglucosaminidase	0.688	0.284	1.21	0.52	−1.02	−2.06	−0.01	antagonistic
	glx	0.279	**0.028**	1.62	0.00	−1.11	−2.15	−0.07	antagonistic
	mnp	0.903	0.966	1.12	1.19	−1.17	−2.21	−0.13	antagonistic
Signal intensity of N cycling genes	NirB	0.907	0.274	1.33	1.01	−1.47	−2.52	−0.41	antagonistic
	napA	0.218	0.884	2.02	0.92	−1.27	−2.31	−0.22	antagonistic
	hzo	0.722	0.454	1.25	0.60	−1.00	−2.06	0.01	–
	narG	0.448	0.258	0.84	0.70	−0.81	−1.84	0.22	–
	nirK	0.186	0.113	1.69	0.55	−1.10	−2.14	−0.06	antagonistic
	norB	0.554	0.280	1.89	1.03	−1.84	−2.91	−0.76	antagonistic
	nosZ	**0.009**	**0.043**	1.79	1.21	−1.48	−2.53	−0.42	antagonistic

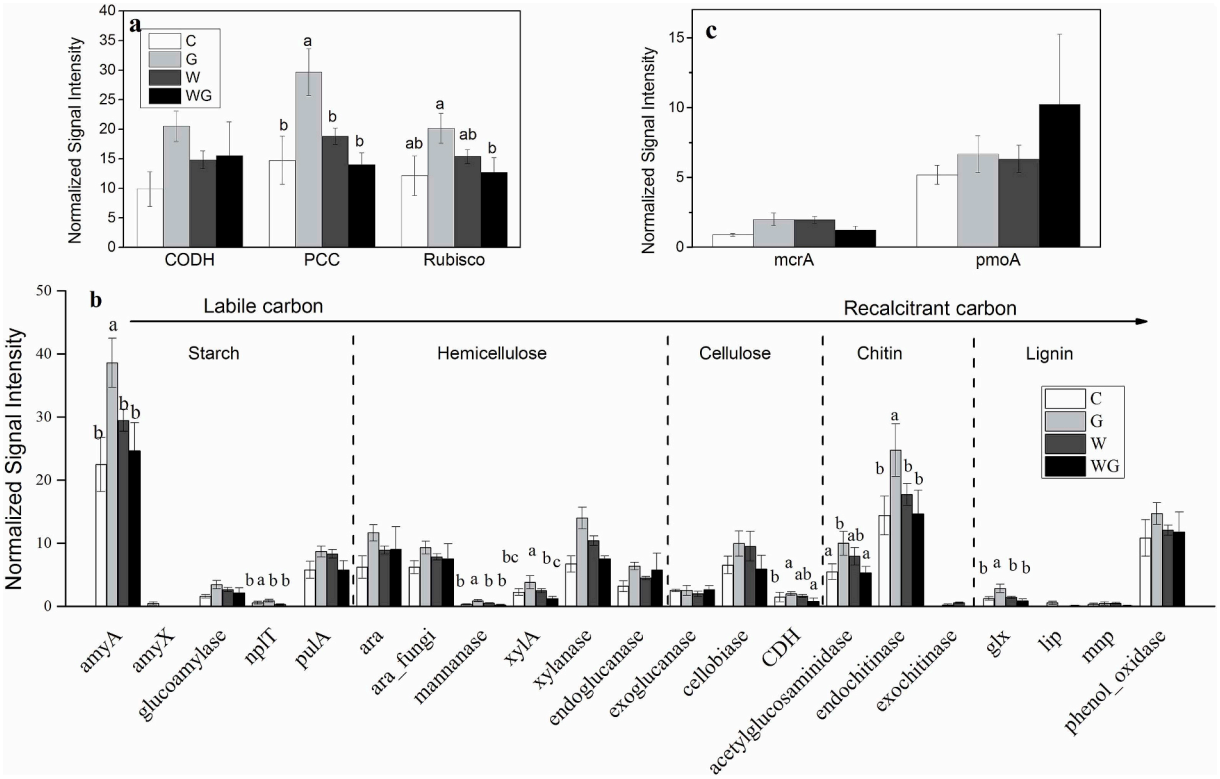

Fig. 3. Normalized signal intensities of functional genes involved in CO_2 fixation (a), carbon-degradation (b), as well as methane production and oxidation (c), in treatments of grazing alone (G), warming alone (W), warming combined with grazing (WG), and control (C). Error bars represented standard errors (n = 4). Different letters above bars indicated significant differences among treatments ($P < 0.05$), determined by *post-hoc* lsmeans tests following Two-way ANOVA.

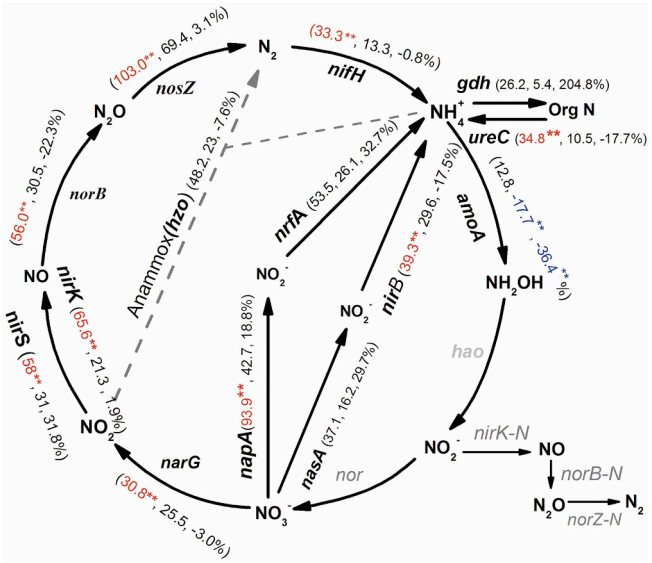

Fig. 4. Percentage changes of signal intensities for detected genes involved in N cycling. The numbers in parentheses from left to right represented changes of grazing alone (G), warming alone (W) and warming combined with grazing (WG) compared to control (C), respectively. Red-coloured numbers indicated significant increases of gene signal intensities in corresponding treatments compared to the control. Blue-coloured numbers indicated significant decreases of gene signal intensities in corresponding treatments compared to the control. Grey-coloured genes were not detected by present GeoChip. The significances were determined by *post-hoc* lsmeans test following Two-way ANOVA and labelled as ** when $P < 0.05$.

3.5. Relationships between microbial community functional structure and environmental variable

RDA (Fig. 5a) revealed that the overall microbial functional community structure was significantly ($P = 0.036$) associated with soil soluble organic N (SON), soil soluble organic C (SOC), coverages of *Carex scabrirostris* and coverages of forbs, belowground root biomass (Biomass*B*), and soil temperature (Soil T). These soil and plant variables explained 71.2% of the variation in soil microbial functional community (Fig. 5a).

Considering that warming and grazing may affect microbial functional structure through different mechanisms, we performed separated constrained ordination analyses with treatment pairs of grazing alone and control, warming alone and control, as well as the combined treatment and grazing alone. RDA with samples from grazing alone and control revealed that plants factors consisting of coverages of all legumes, coverages of *Gueldenstaedtia diversifolia* and plant species richness differentiated treatments and accounted for 77.2% of total variation (RDA model $P < 0.01$). The first and second axis explained 65.7% and 7.6% of total variation, respectively (Fig. 5b). For RDA with samples from warming alone and control, functional structure was marginally significantly ($P = 0.057$) correlated with aboveground biomass (Biomass*A*) and soil C/N. These two factors accounted for 27% of overall variation (RDA model $P = 0.020$; Fig. 5c). For RDA with samples from the combined treatment and grazing without warming, the soil variable (total phosphorous, $P = 0.073$) and vegetation variables (i.e. coverages of forbs, $P = 0.070$ and coverages of *Poa pratensis*, $P = 0.014$) were significantly (RDA model $P = 0.036$) correlated with the functional structure and accounted for 82.9% of variation in functional community composition (Fig. 5d).

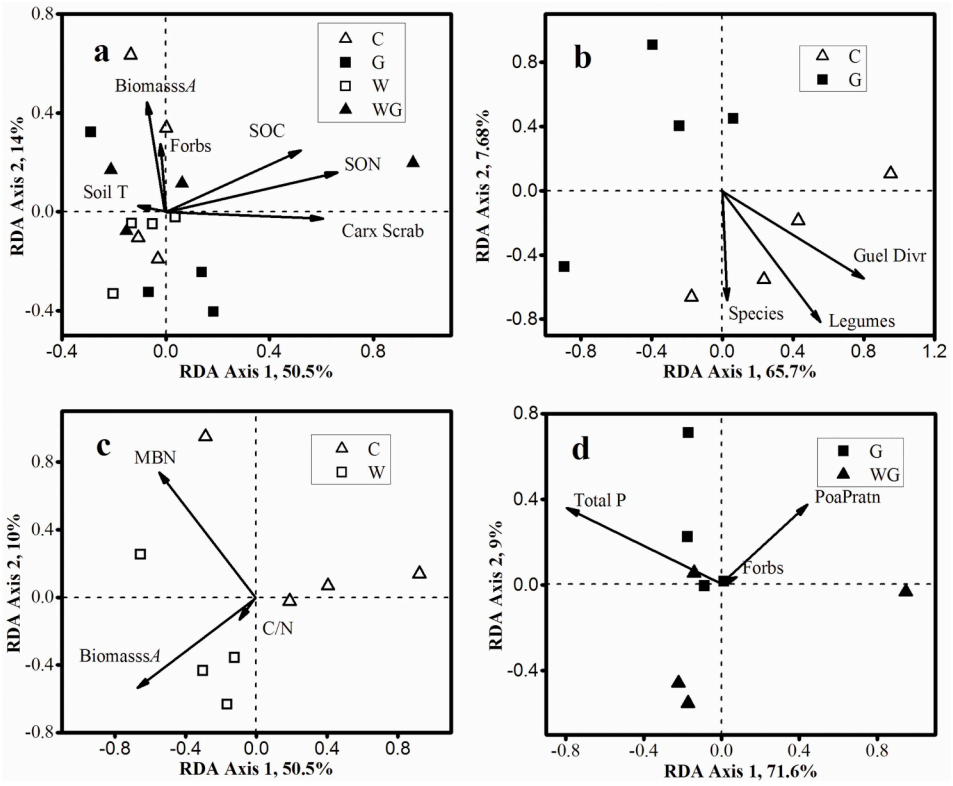

Fig. 5. Redundancy analysis (RDA) based on detected functional genes and selected environmental variables for all samples (a), grazing alone (G) and control (C) samples (b), warming alone (W) and control samples (c), warming combined with grazing (WG) and control samples (d). The percentage values of Axis 1 and 2 were percentage of variations explained by the corresponding axes. Selected environmental variables included soil temperature (Soil T), belowground biomass (Biomass*B*), coverages of forbs (Forbs), soil organic carbon (SOC), soil organic nitrogen (SON), coverages of *Carex scabrirostris* (CarxSscab), plant species richness (Species), coverages of legumes (Legumes), coverages of *Gueldenstaedtia diversifolia* (GuelDivr), microbial biomass nitrogen (MBN), aboveground living present biomass (Biomass*A*), soil carbon: nitrogen ratio (C/N), total phosphorous (Total P), coverages of *Poapratensis* (PoaPratn).

4. Discussion

Many studies reported that grazing alone without warming and warming alone without grazing can alter the soil microbial taxonomic composition and structure (Clegg, 2006; Banning and Murphy, 2008; Ingram et al., 2008) and abundances of microbial functional genes (Xie et al., 2014; Pan et al., 2018), e.g. *amoA* and *nirK*, in alpine grassland ecosystems (Xie et al., 2014). In this study, by using Geochip, we examined functional metabolic potentials of soil microbial communities under warming and grazing in an alpine meadow.

4.1. Effects of grazing on soil microbial functional communities

Compared to the control, grazing alone significantly changed overall functional gene structure and increased alpha diversity, consistent with previous studies in alpine meadows adopting Geochip technology as well (Yang et al., 2013; Wang et al., 2016). Grazing alone also significantly increased the microbial functional potentials in C degradation and fixation, N fixation, mineralization and denitrification. The stimulating effect of grazing alone on microbial C and N genes were conflicted with those results obtained from the semiarid grasslands. In semiarid grasslands, grazing-inhibited C/N cycling processes were observed and related to the reduction in soil moisture or soil C and N contents (Phetteplace et al., 2001; Wang et al., 2006; Xu et al., 2008), which were not observed in this study. In the alpine meadow, decreased C degradation genes were previously observed from a free livestock grazing study wherein heavy grazing occurred (Yang et al., 2013), and such a discrepancy could be explained by the fact that a lower grazing intensity was adopted in our study, and different grazing intensities can have opposite effects on C/N pools and fluxes (Zhou et al., 2017). For example, soil C sequestration under low-intensity grazing may change to C loss under heavy grazing in a temperate grassland (He et al., 2011).

Our result was in line with many studies in grazed pastures, located in New Zealand (Menneer et al., 2005) and Europe (Chronakova et al., 2009), where accelerated C and N cycling processes were induced by moderate grazing as well. In the alpine meadow, a stimulatory effects of moderate grazing on *nifH* gene for N fixation (Che et al., 2018), *ureC* gene for ammonification (Xu et al., 2011) and denitrification genes (Xie et al., 2014) were also reported. The stimulated C and N cycling processes or function genes under grazing were likely due to enhanced soil C and nutrient contents (Saggar et al., 2004; Oenema et al., 2007; Keil et al., 2011). In this study, grazing alone did not change the measured soil C or N contents in the depth of 0–20 cm. However, total inorganic N at the 0–10 cm depth (Rui et al., 2011) was observed to be significantly increased by grazing alone. It is also possible that urine and dung deposition from animals in grazed plots may increase the labile portion of substrate input into soils, posing stimulating impacts on microbial function genes involved in C and N cycling. These phenomena might also be explained by plant community shift as grazing alone significantly decreased coverages of *Gueldenstaedtia diversifolia* and total coverages of legumes (Table S2), explaining 77.2% of variances in microbial functional structure by RDA.

4.2. Effects of warming on soil microbial functional communities

In our study, short-term (4-year) warming alone did not change functional gene alpha diversity, functional community structure and abundances of most C and N cycling genes (except *amoA*). Only *nplT*, manganese gene and *glx* for C decomposition, PCC for C fixation and *nosZ* for nitrous oxidation were significantly negatively affected by the main effect of warming, inconsistent with unchanged abundances of some of these genes (i.e. *nplT and glx*) in the tallgrass prairie ecosystem (Yue et al., 2015; Cheng et al., 2017). Insignificant warming effects for most functional genes were also observed in some previous studies on species composition of soil microbial community in stenothermal (Zhang et al., 2005; Gray et al., 2011) or alpine ecosystems (Li et al., 2016; Zhang et al., 2016) under short-term warming experiments (less than 4-year warming treatment) with similar temperature increase. However, inconsistent with our results, Yue et al. (2015) found that abundances of C and N cycling genes were decreased by short-term (3 years) warming in a Tibetan alpine meadow. The temperature increase in their warming treatment reached approximate 5 °C, much higher than ours (1.2–1.7 °C), likely explaining such conflict. On the other hand, numerous studies found substantial changes in soil microbial community properties under long-term warming treatments (Luo et al., 2014; Pold et al., 2016; Cheng et al., 2017). Therefore, distinct observations might be attributed to a long time lag between climate changes and detectable responses in the microbial community caused by slow incorporation of plant litter into large soil organic C pool, and temperature increase extents (Rinnan et al., 2007; Weedon et al., 2012; Yergeau et al., 2012; Streit et al., 2014; Xu and Yuan, 2017). In our study, soil substrate was not affected by warming, which might be explained by the balance between increased soil substrate input from plant litter and exudates Vs. increased aboveground plant uptake. Thus, the stable soil substrate likely resulted in unchanged microbial functional diversity and structure. The more sensitive response of *amoA* than other N genes in nitrification and denitrification was consistent with studies from temperate grasslands (Zhang et al., 2013, 2017). Decreased *amoA* abundance could be explained by inferior competitiveness for nutrient of associated microbes due to chemoautotroph (Belser, 1979; Kowalchuk and Stephen, 2001).

4.3. Interactive effects between warming and grazing on soil microbial communities

Our study showed that warming greatly dampened the effects of grazing on microbial function gene diversity, community structure and C/N gene abundances, resulting in insignificant effects of the combined treatment. Warming and grazing influenced plant community differently, which may contribute to the dampening effect in the combined treatment. For example, grazing alone significantly decreased aboveground plant biomass and the coverage of legumes, but warming alone increased them (Table S2). Warming alone significantly reduced the plant diversity, while grazing alone did not (Tables S1 and S2). The opposite or different effects of grazing and warming on plant community could strongly affect microbial functional community through changing root exudates, litter, and thus soil substrate availability (Stephan et al., 2000; Bardgett, 2011), resulting in insignificant differences in soil functional genes between the combined treatment and control.

Almost all concentrations of soil nutrients were highest in the combined treatment (Table S2), including significantly increased soil total inorganic N and NO_3^--N, possibly due to decreased aboveground plant biomass with less nutrient uptake but increased belowground plant biomass with more soil C input. Though the combined treatment had significantly higher soil NO_3^- -N content, similar to the grazing alone treatment, no significant effect of the combined treatment was observed on microbial functional properties. Insignificant effects of the combined treatment on microbial functional properties was possibly due to limitation by water deficit as revealed by the significantly decreased soil moisture content, mainly posed by warming effect. Considering that in 2009, a drought year (Hu et al., 2010), the decrease in soil moisture could limit the physiological activity of soil microorganisms. Moreover, previous studies in the same experimental site have shown that short-time warming significantly increased dung mass loss (Luo et al., 2010), likely inhibiting the stimulating effect of urine and dung deposition from animals in the combined treatment as well. As a result of such dampening effects, significant interactions between warming and grazing on microbial function genes were mainly antagonistic (Table 2).

The active component or gene expression (RNA) is widely recognized to be more related to actual functions, but more sensitive to

environmental changes and considered to be easily affected by conditions during the sampling time and other disturbances (Che et al., 2016; Xue et al., 2016a). It would be beneficial to investigate the microbial community by multiple techniques to characterize different aspects and acquire in-depth understanding of soil microbial functioning in alpine ecosystems in further studies.

5. Conclusions

To our knowledge, our study represents the first evidence indicating that grassland management regime and climate change could interact antagonistically on soil microbial functional groups in high-altitude ecosystems, which may have important implications for predicting future soil C and N processes. Soil microbial community structure and functional genes remained unchanged under moderate grazing in warming scenarios compared to the control, despite enriched soil nutrient availability. Such phenomena may be attributed to a warming-induced water deficit, which may dampen the stimulating effect of moderate grazing on microbial functional properties in the experimental site we studied. Therefore, the assessment of grazing effects on soil carbon/nitrogen processes in the Tibetan Plateau alpine meadows should take climate warming into consideration in future scenarios.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.02.018.

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