Evaluating the lingering effect of livestock grazing on functional potentials of microbial communities in Tibetan grassland soils

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Abstract

Background and aims: Livestock grazing is a widely practiced land-use regime that can impose lingering effects on global biogeochemical cycles. However, elucidating the mechanisms of related eco-processes, which are largely mediated by the microbial community, remains challenging. *Methods*: Here, we collected soil samples from two Tibetan grassland sites subjected to grazing in winter followed by a 3-month recovery. We then evaluated functional potentials of microbial communities via a metagenomic tool known as GeoChip 4.0. *Results*: Significant alterations were detected in post-grazing grassland soils, and further analysis showed that plant diversity was the best indicator of alterations in functional potentials. Relative abundances of labile C degradation genes decreased at the 3400-m site, but those of recalcitrant C degradation genes increased, which could be explained by the higher soil recalcitrant C input owing to their being substantially more forbs species at this site. Nitrification genes decreased at both sites, probably owing to increased soil moisture conducive to oxygen-limiting conditions. Relative abundance of denitrification genes increased at the 3200-m site, concomitant with increased N_2O emissions. *Conclusions*: These results demonstrated that functional gene compositions of the microbial community were altered in post-grazing grassland soils, and linked to soil biogeochemical processes.

Keywords: Microbial functional potential, Tibetan grassland, Post-winter grazing, GeoChip

Introduction

Being the third largest ice store after Antarctica and the Arctic (Qiu 2008, 2010), the Tibetan Plateau is among the regions suffering the most from global warming and anthropogenic perturbation. Livestock grazing is a dominant economic activity in the Tibetan Plateau (Cao et al. 2004); however, it imposes marked and lasting impacts on above- and below-ground ecosystems of land surfaces via a number of mechanisms such as trampling, defoliation, defecation, and urination (Bardgett and Wardle 2003; Hamilton and Frank 2001; Radl et al. 2007; Ruess and Mcnaughton 1987). As a result, the magnitude of soil carbon (C) and nitrogen (N) pools could be altered by reallocating C and N between roots and shoots, or by recycling C and N through the animal excreta pathway (Hamilton and Frank 2001; Ruess and Mcnaughton 1987). Soil C stock has been shown to decrease significantly in temperate grassland during 20 years of grazing, which might be owing to loss from respiration (Schipper et al. 2007). However, another analysis found that C stock was unchanged in grazed tussock grassland and increased in grazed hill country soil (Schipper et al. 2010). Currently, the reasons for such inconsistencies in changes in soil C stock are not clear.

Soil microbial communities are essential to mediating biogeochemical cycles of C and N (Carney et al. 2007; van Groenigen et al. 2006). The DNA copy number of the methyl coenzyme M reductase (*mcrA*) gene increased immediately in response to grazing, concurrent with an increase of methane (CH_4) emissions (Radl et al. 2007). Additionally, grazing increased the abundance of the ammonia-oxidizing gene (*amoA*), which was attributed to increased conversion of urea into ammonia in animal excrement (Radl et al. 2014; Xie et al. 2014). The ratio of ammonia-oxidizing bacteria (AOB) to archaea (AOA) also increased because AOB was more adaptive to alkaline pH and higher ammonia concentrations (Radl et al. 2014; Xie et al. 2014). Both denitrification potentials and nitrous oxide (N_2O) emissions increased in response to grazing. This occurred because major factors controlling denitrification, such as nitrate (NO_3^--N), C, soil moisture, and soil compaction, increased, which led to increased nutrient input and oxygen-limiting conditions favoring denitrification (Chroňáková et al. 2009; Hynšt et al. 2007).

However, knowledge base regarding grazing impacts on environment and biota responses is far from adequate because of the extreme complexity of microbial communities, differences among various ecosystems, and limitations of conventional approaches for characterizing functional potentials of microbial communities. To address these shortcomings, we recently studied grazing effects on soil microbial communities at two sites in Tibetan grasslands (Yang et al. 2013). Specifically, we used GeoChip 4.0, a high-throughput microarray-based metagenomics tool, to evaluate microbial

community functional potentials (Chu et al. 2014; Sun et al. 2014). We found that overall functional gene compositions of the soil microbial community were sensitive to grazing. Relative abundances of C degradation genes decreased, likely as a result of vegetation removal by grazing. In addition, the abundance of N mineralization and nitrification genes increased, but that of denitrification genes decreased, which may have been caused by the return of animal excreta N as substrate. The abundance of microbial genes associated with antibiotic resistance and virulence also increased, which could be attributed to the presence of livestock.

Although our previous study provided valuable insight into soil microbial community responses to grazing at the functional gene level, it focused only on the immediate effects of the grazing regime. However, grassland recovery after grazing is a common land-use management strategy in many places around the world. Grassland recovery has been shown to fully restore plant growth and certain eco-processes (e.g., CH_4 emissions) if grazed at moderate intensity, but grazing at high intensity leads to soil deterioration (Cao et al. 2004; Pappas and Koukoura 2013; Radl et al. 2007). Therefore, it is necessary to examine functional potentials of microbial communities in post-grazing grassland soils. To accomplish this, we conducted a metagenomics study in a post-grazing Tibetan alpine grassland with GeoChip 4.0 three months after open free grazing from November to May.

In this study, we specifically address the following scientific questions: (1) Is there any alteration of functional potentials of microbial communities in post-grazing grassland soils? (2) Which environmental attributes influence functional potentials? (3) Can changes in microbial functional genes explain changes in C and N cycles?

Materials and methods

Site description and sampling

The experiment was conducted at the Haibei Alpine Meadow Ecosystem Research Station, situated at latitude 37°37′ N, longitude 101°12′ E of the Tibetan Plateau, China. This station has a typical plateau continental climate with a mean annual temperature (MAT) of −2 °C and a mean annual precipitation (MAP) of 500 mm (Zhao and Zhou 1999). The soil is a clay loam classified as Gelic Cambisols, with relatively thin soil horizon and high soil organic matter (SOM) contents, which is typical of alpine meadow soil in the Tibetan Plateau. As shown in a previous study (Luo et al. 2009), soil pH of the study site is 7.3–7.4, as determined in saturated paste with 1:5 (*v*/v) soil:H_2O extracts. Soil bulk density is 0.75 g/cm^3 and 1.11 g/cm^3 at a depth of 10 cm and 20 cm, respectively, which was measured using a core sampling method.

The experiments were carried out at two sites within the experimental station (we named the sites according to their elevations, for convenience). The dominant vegetation species at the 3200-m site are *Kobresia humilis*,

Elymus nutans, *Festuca ovina*, *Scripus distigmaticus*, *Potentilla anserine*, and *Gentiana straminea*. The dominant vegetation species at the 3400-m site are *Festuca ovina*, *Stipa capillata*, *Polygonum viviparum*, *Aster tataricus* and *Ligularia virgaurea*. Both sites have decades-long history of free, open livestock grazing from November to May but closed in the growing season of May–October for vegetation recovery. In May 2006, we established three fenced plots of 1.0 m × 1.0 m size with wire (height of 1.2 m at the 3200-m site and 1.7 m at the 3400-m site) as controls to block livestock grazing, and three adjacent, open plots of 1.0 m × 1.0 m size for free livestock grazing.

Soil samples were collected in August 2009. Each sample was composited from five soil cores (diameter of 1.5 cm) collected at a depth of 0–20 cm from each plot. Soil samples were placed on ice and immediately transferred to the laboratory, where visible plant roots and rocks were removed, samples were sieved through 2 mm mesh and completely homogenized prior to storage at 4 °C for soil attributes measurements and −80 °C for DNA extraction.

Soil and plant attribute measurements

Soil temperature was measured at a depth of 10 cm using type-K thermocouples (Campbell Scientific, Logan, UT, USA) coupled with a CR1000 data logger. Soil moisture was measured using a frequency domain reflectometer (Model Diviner-2000, Sentek Pty Ltd., Australia). To measure TOC and TN, a TOC-5000 A analyzer (Shimadzu Corp., Kyoto, Japan) and a Vario EL III Elemental Analyzer (Elementar, Hanau, Germany) was used, respectively. NH_4^+-N and NO_3^--N were measured as previously described (Yang et al. 2013). Briefly, 10 g dry soil was suspended in 50 mL of 2 M KCl solution, shaking for 1 h at the speed of 250 r/min and subsequently standing for 30 min at room temperature. The supernatant was filtered through a cellulose medium speed filter of 30–50 μm pore size, and then concentrations of NH_4^+-N and NO_3^--N were measured with an automated chemistry analyzer (FIAstar 5000 Analyzer, FOSS, Hillerd, Denmark).

Greenhouse gases (GHGs), including carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), were measured essentially as previously described (Lin et al. 2009). In brief, GHGs were measured using opaque, static chambers every 7–10 days during the growing season from May to September in 2009. Chambers were set at a depth of 10 cm at each plot, and the fluxes were measured between 9 and 11 a.m. local time when fluxes were equivalent to one-day average flux. Chambers were closed for half an hour and gas samples were collected every 10 min using airtight syringes to evaluate the rate of changes in GHGs concentrations. Concentrations of GHGs were quantified by an Agilent 4890D gas chromatography (Agilent Co., Santa Clara, CA, USA) coupled with a flame ionization detector for CH_4 and CO_2 analysis and an electron capture detector for N_2O analysis.

Plant indices including plant species number and plant biomass were recorded as described by Klein (Klein et al. 2007). A quadrat in the plot was

used. In brief, the mean height and coverage of vegetation canopy were measured using a 1.0 × 1.0 m quadrat divided into 100 0.1 × 0.1 m squares each plot. The plant species were recorded for each quadrat. The height of each plant was recorded for each species by means of 0.1 cm marks along a vertical ruler held behind the pin in a way that did not disturb the vegetation. Average canopy height of each vegetation type within a quadrat was calculated as the average of all the occurring species. Plant aboveground biomass (PAB) was regressed against total canopy coverage and mean canopy height using the data from all of the plots. The equation is: $PAB = -5.2911 + 4.7865C + 3.3751H$ ($r^2 = 0.89$, $n = 168$, $P < 0.001$), where C is total canopy coverage and H is mean canopy height. Plant diversity was calculated based on the formula of Shannon index, as described in a previous study (Yang et al. 2013). Plant species could be separated into different groups based on animal selection: palatable species, including graminoids, sedge and leguminous species, less palatable forbs species and unpalatable noxious forbs species. Detailed plant information was shown as Supplementary Table S1 online.

GeoChip experiment

A Fast DNA Spin kit (MP Biomedical, Carlsbad, CA, USA) was used to extract DNA from 0.5 g soil, following the manufacturer's instructions. Purified DNA was dissolved in nuclease-free water. DNA quality was examined by the ratios of $A_{260/280}$ and $A_{260/230}$ using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA concentrations were examined using the PicoGreen method (Ahn et al. 1996).

GeoChip experiments were carried out as previously described (Liu et al. 2015; Zhao et al. 2014). Briefly, 2 µg DNA was labeled by random primed Cy-5 dye and purified with a QIA quick purification kit (Qiagen, Valencia, CA, USA). After drying in a SpeedVac (ThermoSavant, Milford, MA, USA) for 45 min at 45 °C, labeled DNA was suspended in 120 µL hybridization solution. Then DNA was hybridized with GeoChip 4.0 on a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA) for 16 h at 42 °C. After purification, microarrays were scanned by a NimbleGen MS2000 scanner (Roche, Madison, WI, USA). Signal intensities were subsequently quantified by ImaGene software (Biodiscovery, El Segundo, CA).

Data analyses

Raw data obtained from ImaGene was submitted to the Microarray Data Manager (http://ieg.ou.edu/microarray/) and analyzed using the data analysis pipeline (Yang et al. 2014). Briefly, raw data was processed as following: (i) poor-quality spots were removed, which were flagged or with a signal to noise ratio less than 2.0; (ii) at least two valid values of three biological replicates were required for each probe; (iii) relative abundance normalization was applied to all data; and (iv) natural logarithmic transformation was used before statistical analysis.

We performed two-way ANOVA to investigate interaction between post-grazing and sites. Detrended correspondence analysis (DCA), an ordination technique that uses detrending to remove the arch effect commonly found in the correspondence analysis (Hill and Gauch 1980), was used to determine overall functional gene compositions of soil microbial communities. The dissimilarity test of *adonis*, a permutational multivariate analysis of variance, was used to test the significance of changes in plant and microbial communities. All the detected functional probes were used to profile overall functional gene compositions of microbial communities, and to calculate the alpha diversity of functional genes. The detected gene categories were listed in Table S2. Simple Mantel tests were performed to establish linkages between microbial communities and environmental (soil or vegetation) attributes. To control co-varying effects between environmental attributes, partial Mantel tests were also performed where environmental attributes except the tested one were used as the third matrix. For both tests, Bray-Curtis coefficient and Euclidean distance were used to construct dissimilarity matrices of microbial communities and environmental attributes, respectively. Environmental attributes were standardized by scaling to means of 0 and variances of 1 before constructing dissimilarity matrices. *P* values of Mantel tests were adjusted by multiple testing of FDR correction (Benjamini and Yekutieli 2001). In addition, Pearson correlation was carried out to correlate vegetation attributes (biomass, species and diversity) and microbial functional diversity. All these analyses were performed by functions in the vegan package (v.2.1–1) in R v. 2.13.2.

Results

Overall changes in soil attributes, vegetation, and functional potentials of microbial communities

We examined two alpine grassland sites at elevations of 3200-m or 3400-m that were subjected to free grazing from November to May followed by a 3-month recovery. The post-grazing effects varied by sites, as revealed by two-way ANOVA (Table 1). Therefore, we analyzed these two sites separately to reveal simple main effect. Soil moisture significantly ($P < 0.05$) increased by 29.7 % and 81.7 % at the 3200-m and 3400-m grazing sites, respectively (Table 2). The C/N ratio also significantly ($P < 0.01$) increased at both sites. However, substantial differences between these two sites were notable. Soil Total organic C (TOC) and N (TN) contents decreased significantly ($P < 0.05$) at the 3200-m site, but increased at the 3400-m site. Ammonium (NH_4^+-N) significantly decreased ($P = 0.03$), and N_2O efflux increased significantly ($P = 0.03$) at the 3200-m site.

Table 1 Repeated measures analysis of two-way ANOVAs of post-grazing and site effects on environmental attributes

Environmental attributes	Post-grazing	Site	Post-grazing × Site
Soil temperature	1.00	**0.01**[a]	0.34
NO_3^--N[b]	0.55	0.16	0.45
NH_4^+-N	0.53	0.13	**0.02**
TOC	**0.01**	**0.01**	**0.01**
TN	**0.01**	**0.01**	**0.01**
C/N ratio	**0.01**	**0.01**	**0.01**
Soil moisture	**0.01**	0.81	**0.01**
Plant biomass	**0.01**	**0.01**	**0.01**
Plant species	**0.02**	0.26	**0.01**
Plant diversity	**0.01**	**0.01**	**0.01**
Released CH_4	0.86	0.138	0.59
Released CO_2	0.77	0.604	0.47
Released N_2O	0.08	**0.01**	0.41

[a] Results in the table are *P* values of two-way ANOVAs. Significant values ($P < 0.05$) are shown in bold

[b] Abbreviations: NO_3^- *-N* nitrate, NH_4^+ *-N* ammonium, *TOC* total organic carbon, *TN* total nitrogen

Table 2 Summary of environmental attributes

Environmental attributes	3200-m			3400-m		
	Control	Post-grazing	*P*[b]	Control	Post-grazing	*P*
Soil temperature(°C)	16.30 (0.00) [a]	16.30 (0.00)		8.78 (0.00)	8.78 (0.00)	
NO_3^--N ($g \cdot kg^{-1}$)	0.93 (0.14)	0.95 (0.08)	0.92	0.84 (0.05)	0.67(0.10)	0.28
NH_4^+-N ($g \cdot kg^{-1}$)	3.70 (0.32)	2.43 (0.06)	**0.03**[c]	2.08 (0.44)	2.90(0.12)	0.21
TOC (%)	6.84 (0.02)	6.25 (0.03)	**0.01**	6.30 (0.03)	7.29(0.02)	**0.01**
TN ($g \cdot kg^{-1}$)	5.35 (0.01)	4.62 (0.03)	**0.01**	5.54 (0.02)	5.72(0.04)	**0.03**
C/N ratio	1.28 (0.01)	1.35 (0.01)	**0.01**	1.14 (0.01)	1.27(0.01)	**0.01**
Soil moisture (%)	33.10 (0.00)	42.95 (0.50)	**0.01**	27.21 (0.00)	49.45(1.97)	**0.01**
Plant biomass ($g \cdot m^{-2}$)	432.28 (8.50)	505.72 (4.87)	**0.04**	471.07 (10.81)	325.53(7.90)	**0.01**
Plant species (m^{-2})	22.00 (0.42)	27.93 (1.08)	**0.01**	26.27 (0.71)	25.93(0.66)	0.79
Plant diversity	1.91 (0.05)	2.34 (0.02)	**0.02**	2.58 (0.02)	2.54(0.07)	0.67
Released CO_2 ($mg \cdot m^{-2} \cdot h^{-1}$)	686.58 (42.30)	718.76 (19.14)	0.60	639.74 (36.03)	623.51(38.60)	0.81
Released CH_4 ($\mu g \cdot m^{-2} \cdot h^{-1}$)	−30.02 (6.29)	−32.33 (1.36)	0.78	−36.66 (4.33)	−31.23(3.15)	0.45
Released N_2O ($\mu g \cdot m^{-2} \cdot h^{-1}$)	8.49 (0.62)	16.18 (1.71)	**0.03**	1.96 (1.07)	4.97(3.85)	0.57

[a] Values are means (standard error); $n = 3$

[b] Differences between control and post-grazing grassland samples at each site were examined by two tailed *t*-tests

[c] Significantly ($P < 0.05$) changed values between control and post-grazing grassland samples are shown in bold

The major plant species at the 3200-m site were palatable graminoids (42–57 % of total species), whereas the major plant species at the 3400-m site were less palatable forbs species (40–66 % of the total species) (Table 3). Plant species, biomass, and diversity significantly ($P < 0.05$) increased at the 3200-m site, whereas plant biomass decreased at the 3400-m site (Table 2). In addition, plant community composition was altered (Fig. 1a), and the significance of the difference was demonstrated by the *adonis* dissimilarity test ($R^2 = 0.283$, $P = 0.001$ for the 3200-m site and $R^2 = 0.302$, $P = 0.001$ for the 3400-m site).

Table 3 Plant composition of different functional groups (%)

Functional group	3200-m			3400-m		
	Control	Post-grazing	P[b]	Control	Post-grazing	P
Graminoids species	55.4 (0.02)[a]	47.7 (0.04)	0.14	33.2 (0.05)	21.2 (0.03)	0.13
Sedge species	5.9 (0.02)	16.4 (0.04)	0.08	5.2 (0.02)	7.1 (0.02)	0.50
Leguminous species	6.4 (0.02)	14.2 (0.02)	0.07	4.6 (0.02)	7.5 (0.04)	0.50
Forbs species	16.4 (0.04)	13.2 (0.05)	0.64	46.3 (0.07)	55.3 (0.06)	0.36
Noxious species	15.9 (0.01)	8.6 (0.01)	**0.01**[c]	10.7 (0.02)	8.9 (0.03)	0.63

[a] Values are means (standard error); $n = 3$

[b] Differences between control and post-grazing grassland samples at each site were examined by two tailed t-tests

[c] Significantly ($P < 0.05$) changed values between control and post-grazing grassland samples are shown in bold

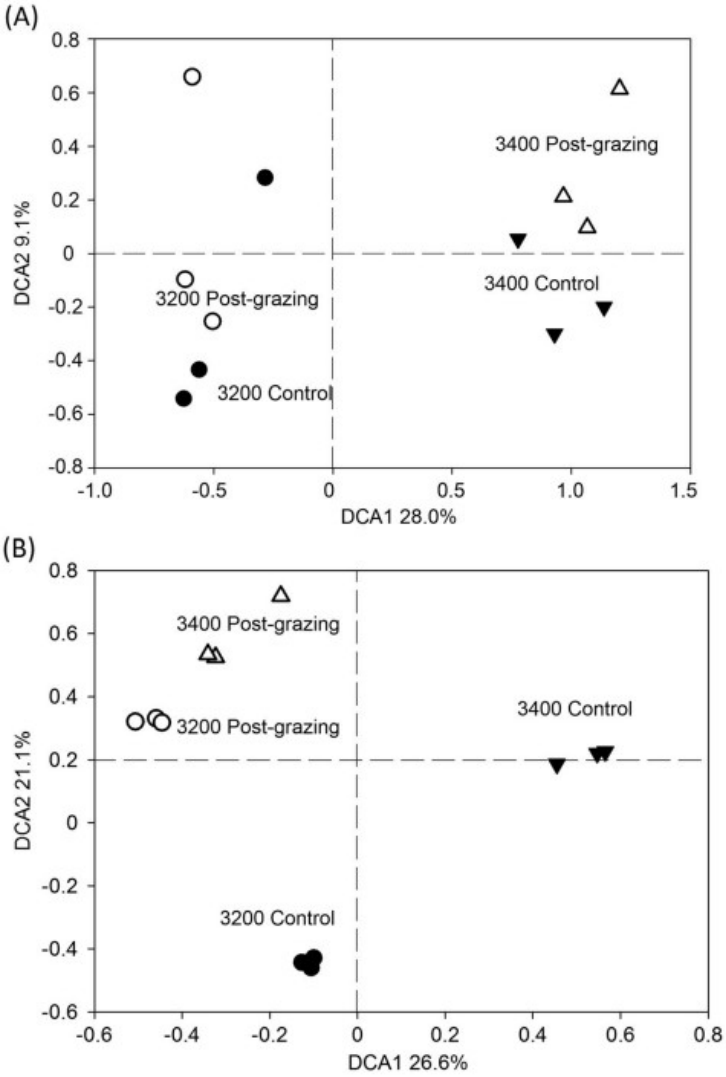

Fig. 1 Detrended correspondence analysis (DCA) of (**a**) plant communities and (**b**) functional gene compositions of microbial communities. Filled and empty symbols represent control and post-grazing grassland samples, respectively. *Circles* and *triangles* represent samples at the 3200-m and 3400-m sites, respectively

The alpha diversity of microbial functional genes, as determined by the Shannon index, marginally significantly ($P = 0.06$) increased at the 3200-m site, while it decreased significantly ($P = 0.01$) at the 3400-m site (Fig. 2a). These findings were similar to changes observed in plant biomass, species number, and diversity (Table 2). DCA showed that functional gene compositions of microbial communities in post-grazing grassland soils were distinct from those in controls (Fig. 1b), which was also verified by statistical analysis conducted using *adonis* ($R^2 = 0.87$, $P = 0.001$ for the 3200-m site and $R^2 = 0.79$, $P = 0.001$ for the 3400-m site). Notably, the beta diversity values of microbial functional genes determined by Bray–Curtis coefficients between post-grazing grassland soils of the two sites were much smaller than those between control soils (see Supplementary Fig. S1A online), suggesting that microbial functions were homogenized. Consistently, samples in post-grazing grassland soils were located in close proximity to the DCA plots (Fig. 1b), while the microbial functional gene

diversities at the two sites (based on the Shannon index) were more similar in post-grazing grassland soils than in controls (Fig. 2a).

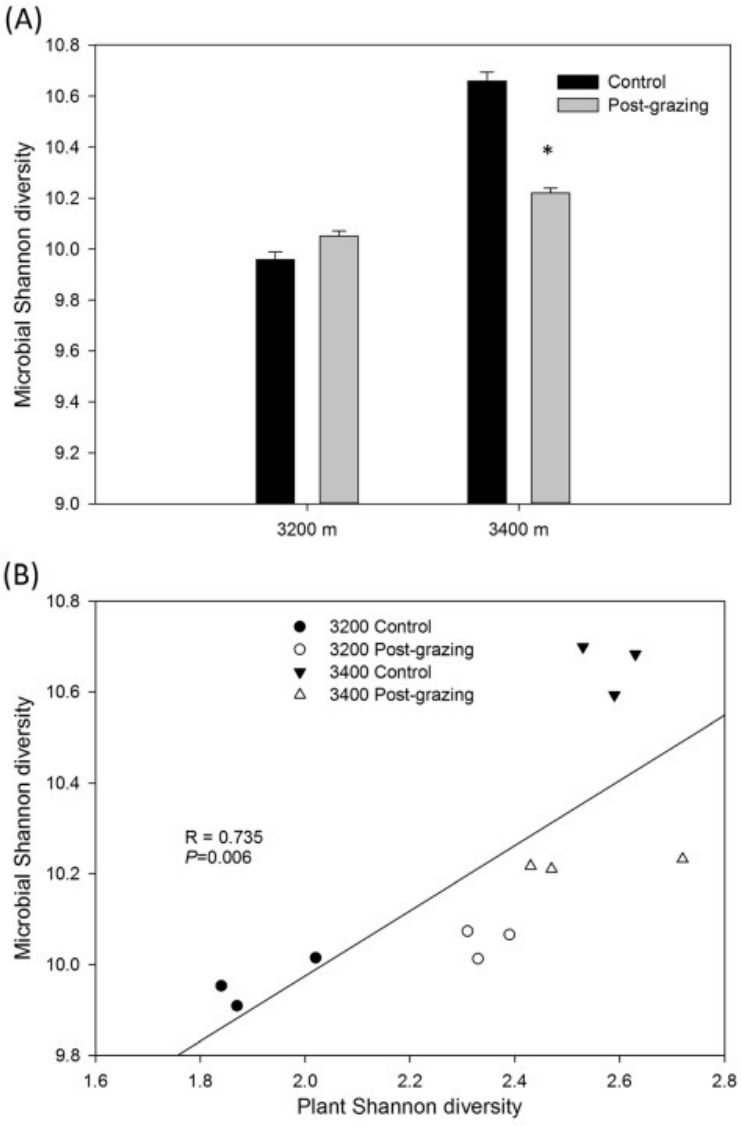

Fig. 2 (**a**) Microbial functional diversities at the 3200-m and 3400-m sites. *Bars* represent the standard errors. Significance was examined by a two-tailed t-test: '*' $P < 0.05$; '**' $P < 0.01$. (**b**) Pearson correlation between microbial functional diversity and plant diversity

Major environmental attributes influencing microbial community

Pearson correlation analysis revealed a strong ($R = 0.74$, $P = 0.01$) correlation between microbial alpha diversity and plant alpha diversity (Fig. 2b). To verify this, we conducted simple Mantel tests to unveil major environmental attributes that influenced overall functional gene compositions of microbial communities. We found that plant diversity was the best indicator of changes in functional gene compositions among the measured attributes, as shown by a strong ($R = 0.70$, $P = 0.007$) correlation (Table 4). When the other environmental attributes were controlled in partial Mantel tests, plant diversity remained the attribute most strongly correlated with functional gene compositions. Closer examination showed that this linkage could be attributed to changes in plant species rather than plant

biomass, as shown by both simple and partial Mantel tests. Meanwhile, simple Mantel tests showed that soil temperature, soil moisture, C/N ratio, and NH_4^+-N contents were also correlated with functional gene compositions, albeit at lower levels than plant diversity. In contrast, total soil organic C was not correlated with functional gene compositions.

Table 4 Relationships between microbial community and environmental attributes revealed by Mantel tests

Environmental attributes	Simple Mantel		Partial Mantel[b]	
	R	*P*	R	*P*
Soil temperature	0.415	**0.016**[a]	0.267	0.074
NO_3^--N	−0.023	0.566	−0.109	0.997
NH_4^+-N	0.265	0.056	0.342	**0.026**
TOC	0.141	0.217	−0.126	0.997
TN	0.239	0.093	0.151	0.223
C/N ratio	0.438	**0.010**	0.365	**0.023**
Soil moisture	0.499	**0.007**	0.477	**0.013**
Plant biomass	0.017	0.430	−0.287	0.997
Plant species	0.431	**0.010**	0.431	**0.022**
Plant diversity	0.700	**0.007**	0.654	**0.013**
Released CH_4	−0.056	0.617	−0.206	0.997
Released CO_2	0.067	0.333	0.043	0.493
Released N_2O	0.150	0.180	0.123	0.249

[a] *P* values were corrected using the FDR procedure. Significant ($P < 0.05$) correlations are shown in bold

[b] The constraining matrix of the partial Mantel test is all of other environmental attributes except the tested attribute

Changes in C cycle genes

A total of 1077 genes were detected in four separate C fixation pathways: ribulose-l, 5-bisphosphate carboxylase/oxygenase, propionyl-CoA/acetyl-CoA carboxylase (PCC/ACC), carbon monoxide dehydrogenase, and ATP citrate lyase (aclB). The PCC/ACC pathway appeared to be the most abundant C fixation pathway for Tibetan grassland soils because it accounted for roughly 61 % of the total C fixation gene abundance. At both the 3200-m and 3400-m sites, the abundance of the *pcc* gene increased in post-grazing grassland soils (see Supplementary Fig. S2 online). Conversely, *aclB*, which accounted for 4 % of the total C fixation gene abundance, decreased, indicative of differential regulation of C fixation pathways.

For C degradation genes, there were changes in individual functional genes (e.g., the increase of *isopullulansase* and *glx* and the decrease of *nplT* and

vdh) at the 3200-m site (Fig. 3a). In contrast, the abundance of *amyA*, *nplT*, *pulA*, *pectinase*, *ara_fungi*, *mannanase*, and *xylA* associated with the degradation of labile C (starch, pectin, or hemicellulose) decreased in post-grazing grassland soils of the 3400-m site, whereas those of *vdh*, *vanA*, *glx*, and *lip* associated with degradation of recalcitrant C (aromatics or lignin) increased (Fig. 3b), suggesting a shift from use of labile C toward recalcitrant C. The *vdh* and *vanA* genes associated with the bioconversion of vanillin to protocatechuate for aromatics degradation were abundant in *Pseudomonas sp.*, which was consistent with the results of a previous study (Priefert et al. 1997). The most abundant *lip* gene was similar to the sequence from *Trametes versicolor*, which was shown to play an important role in lignin oxidation (Bourbonnais et al. 1995; Johansson and Nyman 1993).

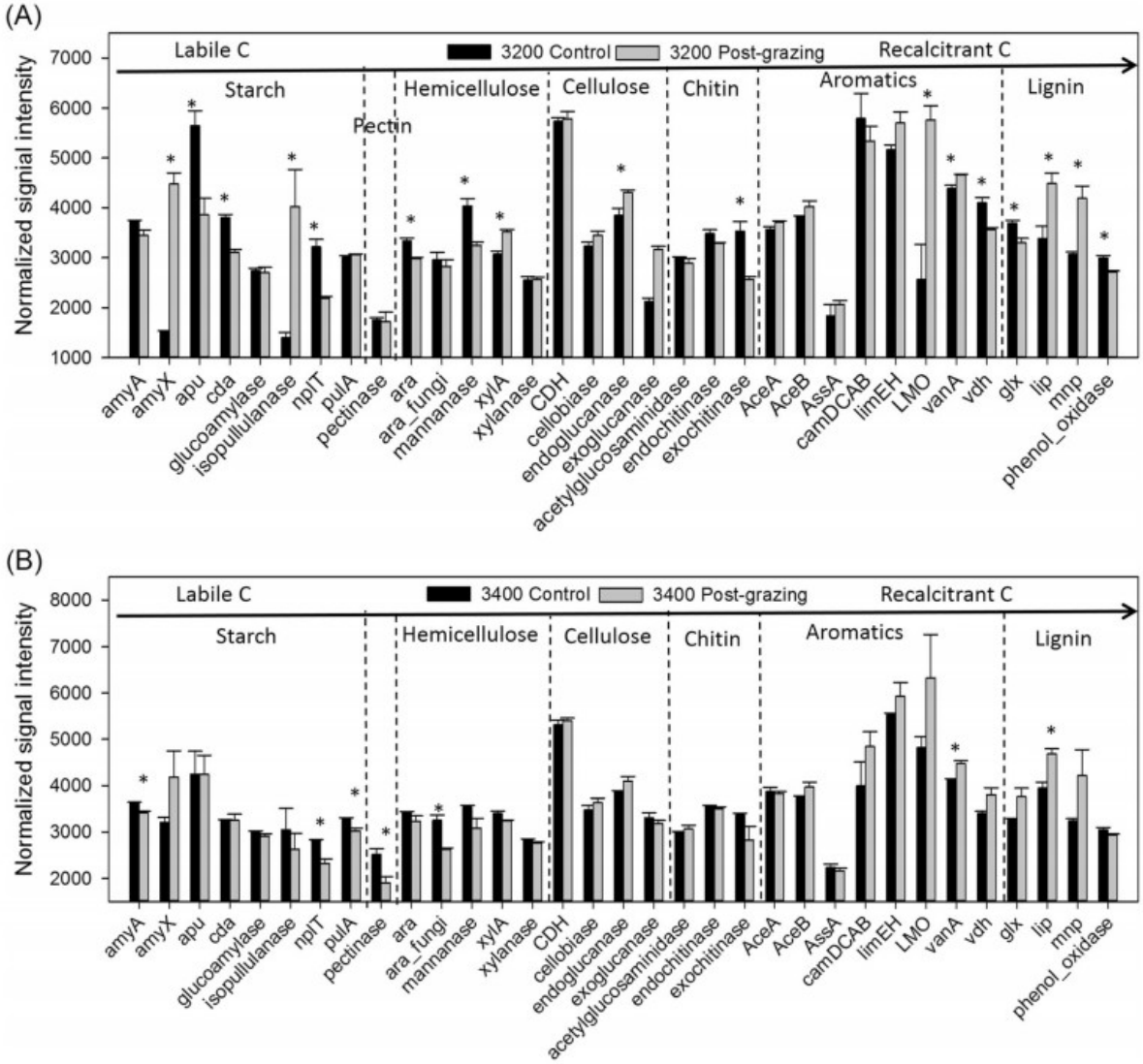

Fig. 3 Normalized signal intensity of functional genes associated with C degradation at (**a**) the 3200-m site and (**b**) the 3400-m site. The normalized signal intensity for each gene was the sum of abundances of detected genes divided by the detected gene number across all samples. *Bars* represent standard errors. Significance was examined by two-tailed *t*-tests: '*' $P < 0.05$; '**' $P < 0.01$

The abundance of the CH_4 oxidation gene, *mmoX*, encoding CH_4 monooxygenase, decreased by 8 % at the 3200-m site and by 25 % at the 3400-m site (see Supplementary Fig. S3 online). In addition, the abundance of another CH_4 oxidation gene, *pmoA*, decreased by 10 % at the 3400-m site, which suggests that CH_4 oxidation might be suppressed. The abundance of the CH_4 production gene, *mcrA*, increased at the 3200-m site, but remained unchanged at the 3400-m site.

Changes in N cycle genes

Because NH_4^+-N contents were correlated with the microbial community (Table 4), we examined N cycle genes more closely. The relative abundance of the *gdh* gene, which encodes glutamate dehydrogenase and converts alpha ketoglutarate and NH_4^+-N to glutamate, increased at the 3200-m site and decreased at the 3400-m site, whereas the relative abundance of the *ureC* gene, which encodes a protein that catalyzes the hydrolysis of urea to NH_4^+-N, decreased at the 3200-m site (Fig. 4). This might have resulted in opposite net effects or a shift between urea and ammonia conversion at these two sites. Concomitantly, the NH_4^+-N content decreased at the 3200-m site and remained unchanged at the 3400-m site. The abundances of *amoA* genes derived from AOA to AOB both decreased at the 3200-m site, whereas AOA marginally significantly ($P = 0.08$) decreased and AOB remained unchanged at the 3400-m site. Notably, the ratio of AOA/AOB decreased significantly ($P < 0.03$) by 20.0 % at the 3200-m site and by 12.1 % at the 3400-m site. The abundances of hydroxylamine-oxidizing genes (*hao*) marginally significantly ($P < 0.09$) decreased at the 3200-m and 3400-m sites.

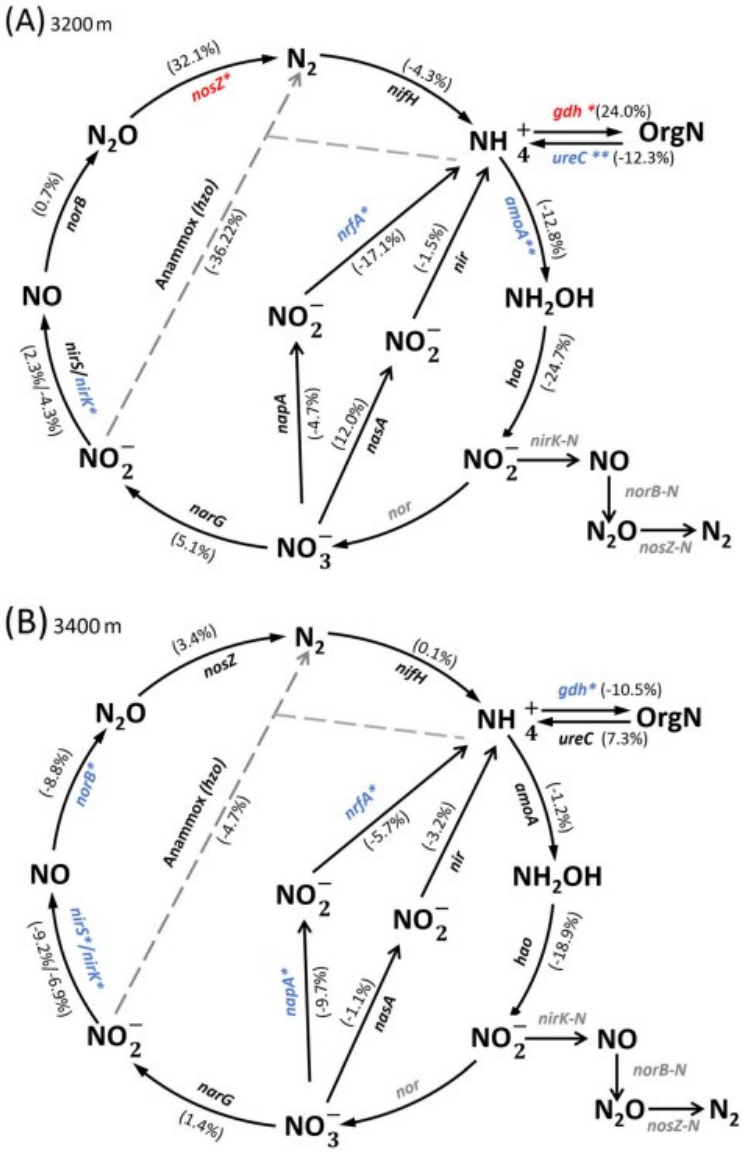

Fig. 4 Normalized signal intensity of functional genes associated with the N cycle at (**a**) the 3200-m site and (**b**) the 3400-m site. The percentages in brackets are fold changes of gene abundances as the sum of probe abundances belonging to the same gene family in post-grazing grassland soils versus control samples. Genes in *red* and *blue* are increased or decreased, respectively. *Gray* genes are those not detected by GeoChip 4.0. Significance was examined by a two-tailed *t*-test: '*' $P < 0.05$; '**' $P < 0.01$

The abundances of the denitrification genes *nosZ* increased at the 3200-m site, which was consistent with the reduction in soil TN content (Table 2). The abundances of denitrification genes *nirS*, *nirK*, and *norB* decreased at the 3400-m site, which could reduce the metabolic potential of N_2O efflux and explain the increase in soil TN content (Table 2). In addition, genes associated with the dissimilatory N reduction pathway (*napA* and *nrfA*) decreased, although this decrease was not significant for the 3200-m site.

Changes in genes associated with complex C metabolism

Anthropogenic activities might have introduced complex C compounds to our study sites. We found that the abundances of genes associated with complex C metabolism significantly ($P = 0.004$) increased at the 3200-m site (see Supplementary Fig. S4A online). Moreover, the abundances of these genes increased at a less degree at the 3400-m site, which coincidently had less

local residence and anthropogenic activities. Among these, the increase of genes associated with biodegradation of aromatics ($P = 0.01$), herbicide-related compounds ($P = 0.01$), and other organics ($P = 0.04$) was most notable at the 3200-m site (see Supplementary Fig. S4B online). For example, gene abundances required for degradation of aromatic alpha hydroxy acid, aromatic carboxylic acid, chlorinated aromatics, and nitroaromatics increased by 18.8 %, 7.1 %, 10.9 %, and 6.2 %, respectively. Similarly, genes associated with the degradation of herbicide-related compounds, such as *atzB*, *atzC*, and *trzN*, also increased. These genes were most similar to sequences from *Bradyrhizobium* sp., *Burkholderia phymatum*, *Nocardioides* sp., *Rhodococcus* sp., and *Xanthobacter autotrophicus*, which are well known to play important roles in the microbial metabolism of herbicide compounds (Gonod et al. 2006; Larkin et al. 2005; Parker et al. 2007; Satsuma 2010).

Discussion

Our results provided an in-depth view of the functional potentials of microbial communities in post-grazing grassland soils. An instant grazing effect is the reduction of available forage quantity and plant species richness (Bullock et al. 1994), whereas in this study, total plant biomass increased in the 3200-m post-grazing grassland soils, but decreased in the 3400-m post-grazing grassland soils (Table 2). For the belowground ecosystem, grazing imposed a common effect on soil microbial communities that predominated over site differences, as indicated by the decrease of the microbial C cycling gene abundance and the increase of microbial ammonification and nitrification gene abundance (Yang et al. 2013). This reflected an instant response to the trampling, defoliation, defecation, and urination effects of grazing. In contrast, functional potentials of microbial communities differed in post-grazing grassland soils because the alpha diversity increased at the 3200-m site, but decreased at the 3400-m site (Fig. 2). The disparate responses in functional gene compositions can be attributed to substantial differences in plant community responses at the 3200-m and 3400-m sites (Fig. 1a), or the common observation that microbial responses tend to be more diversified over time (Shade et al. 2013) because our soil collection occurred 3 months after grazing.

The beta diversity of functional gene compositions in post-grazing grassland soils was smaller than those in control soils (see Supplementary Fig. S1 online), which suggests functional homogenization of the microbial community. Phylogenetic compositions of soil bacterial communities were found to be homogenized in the Amazon rainforest after conversion of rainforest to cattle pasture (Rodrigues et al. 2013). It has been proposed that homogenization might be caused by the replacement of ecological specialist species with generalist species and consequently cause reduction of ecosystem resistance or resilience (McKinney and Lockwood 1999; Olden et al. 2004). However, functional homogenization has not been adequately investigated.

Plant diversity was identified as the environmental attribute with the strongest correlation to functional gene diversity and composition (Fig. 2b and Table 4). The grazing effects on plant diversity have been documented in other ecosystems (Milchunas and Lauenroth 1993), and they may occur via altered competition among plant communities for resources (Collins et al. 1998) or by creating environmental heterogeneity (Sommer 2000). Notably, plant diversity was strongly linked to microbial community composition in our previous grazing study (Yang et al. 2013). These findings indicate a common linkage between plant diversity and functional gene compositions, regardless of grazing regime and site differences. Similarly, soil moisture was identified as an important factor contributing to functional gene variations in this study, as well as in our previous grazing study. Coincidently, it was a dominant attribute for mediating plant diversity of grasslands (Yang et al. 2011). The close linkages between soil moisture, plant diversity, and microbial community warrant further study to understand the potential consequences of and general responses to grazing.

GeoChip provides large-scale quantitative information regarding important microbial functional groups, such as C and N cycles, making it possible to link microbial functional genes with system-level soil biogeochemical processes. Our GeoChip data unveiled significant changes in the abundance of genes associated with C degradation, suggesting that soil C storage might be affected. Particularly, the relative abundances of labile C degradation genes decreased at the 3400-m site, but those of recalcitrant C degradation genes increased (Fig. 3b). These findings indicate a possible response to the higher recalcitrant plant litter return to soil because there were substantially more forbs species at this site (Wardle et al. 2004) (Table 3).

Soil CH_4 emissions, which are the net effect of microbial mediation of CH_4 production and oxidation, can be affected by land use changes through modifications in soil texture, bulk density, water status, and chemical features. Grazing induced increases in the number of copies of the *mcrA* gene and soil CH_4 emissions (Radl et al. 2007). In our study, the relative abundance of the CH_4-producing *mcrA* gene increased at the 3200-m site, but CH_4-oxidizing genes decreased at both sites (see Supplementary Fig. S3 online), which might have resulted in increased soil CH_4 emissions. However, our findings showed that soil CH_4 emissions remained unaltered (Table 2). This might have been because of increased soil moisture (Table 2), which decreased CH_4 diffusion and consequently emissions in soil (Liu et al. 2007).

Grazing increased the abundance of microbial genes associated with nitrification (Radl et al. 2014; Xie et al. 2014), reflecting an immediate response to animal excreta. In contrast, nitrification genes in post-grazing grassland soils decreased (Fig. 4), which might be attributed to increased soil moisture that inhibits the oxygen-requiring nitrification process (Luo et al. 1999). Notably, the ratio of AOA/AOB decreased in this and previous grazing studies (Radl et al. 2014; Xie et al. 2014), suggesting that selecting AOB as the major group of nitrification by grazing has a lingering effect.

The quantities of N in the urine and dung patches ranged from 30 to 100 $g \cdot m^{-2}$, which exceeds plant requirements. It has been reported that grazing could cause considerable N loss under warm and moist conditions through leaching and denitrification (Ball et al. 1979). However, grazing reallocates N between aboveground and belowground ecosystems, which could compensate soil N loss. Indeed, a continental survey indicated that grazing increased both the rates of N cycling and soil N content (Wolf et al. 2010). In our study, soil TN contents decreased at the 3200-m site and increased at the 3400-m site (Table 2), which might have resulted from differential microbial mediation of N cycles at different sites. This was particularly clear for denitrification. The increase in the relative abundances of *nosZ* at the 3200-m site and the decrease in the relative abundances of *nirS*, *nirK*, and *norB* at the 3400-m site coincided with different changes in soil N contents. Furthermore, changes in *gdh* and *ureC* abundances could shift urea use toward ammonia assimilation at the 3200-m site (Fig. 4), resulting in a reduced NH_4^+-N content (Table 2). These findings provided mechanistic insights into the coupling between the soil N cycle and concurrent changes in microbial N cycle genes. However, it should be noted that changes in gene abundance are not always in alignment with changes in the nutrition pool because the pool size of soil C and N may be too large and heterogeneous. Although significant changes in metabolic processes may occur, the net budget of soil C or N may still be small when compared with the total soil C or N pool, making it difficult to analyze in short-term experiments (Smith 2004).

This is the first field study to evaluate functional potentials of microbial communities in post-grazing grassland soils. Given that grazing is a widely occurring, dominant economic activity in the Tibetan Plateau (Cao et al. 2004), our finding of disparate responses in soil ecosystems suggests that vegetation restoration in post-grazing soils has more complicated ecological consequences than previously anticipated. However, it is important to note that this study had a limited sampling size and only one time point. Nevertheless, this area is typical of a Tibetan grassland landscape and climate. Because general changes in functional gene compositions of the microbial community are modified by local, regional, and temporal variations (Lin et al. 2010; Su et al. 2006), it would be interesting to expand the observations in this study to other grasslands or conduct time-series experiments to test the generality of these observations.

Conclusion

Grazing is a dominant economic activity for the Tibetan Plateau; thus, it is important to assess its ecological impacts for the purpose of grassland conservation and biodiversity management. This study demonstrated that microbial communities were significantly altered in post-grazing grassland soils. Therefore, assessments of the consequences of land use management on grassland ecosystems should take the microbial community into consideration. Meanwhile, grazing followed by recovery offers a tool for

conservation management, which could improve grassland maintenance if carefully and appropriately executed (Cao et al. 2004; Lempesi et al. 2013; Pappas and Koukoura 2013). The strong coupling of the microbial community, plants, and soil biogeochemical cycles makes it possible to achieve the best ecosystem service by regulating individual components of the grassland ecosystem.

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Data accessibility

GeoChip 4.0 data is available online (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE52425.

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