Global Change Biology

Global Change Biology (2013) **19**, 637–648, doi: 10.1111/gcb.12065

Responses of the functional structure of soil microbial community to livestock grazing in the Tibetan alpine grassland

YUNFENG YANG*, LINWEI WU*, QIAOYAN LIN†‡, MENGTING YUAN*, DEPENG XU*, HAO YU§¶**, YIGANG HU‡††, JICHUANG DUAN‡, XIANGZHEN LI‡‡, ZHILI HE§, KAI XUE§, JOY VAN NOSTRAND§, SHIPING WANG†‡ and JIZHONG ZHOU*§§§

*State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China, †Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau Research, Chinese Academy of Sciences, Beijing 100101, China, ‡Key Laboratory of Adaption and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China, §Institute for Environmental Genomics and Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA, ¶State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China, **College of Resource and Environment Engineering, Liaoning Technical University, Buxin, Liaoning 123000, China, ††Shapotou Desert Experiment and Research Station, Cold and Arid Regions and Environmental & Engineering Research Institute, Chinese Academy of Sciences Lanzhou 730000, China, ‡‡Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China, §§Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*

Abstract

Microbes play key roles in various biogeochemical processes, including carbon (C) and nitrogen (N) cycling. However, changes of microbial community at the functional gene level by livestock grazing, which is a global land-use activity, remain unclear. Here we use a functional gene array, GeoChip 4.0, to examine the effects of free livestock grazing on the microbial community at an experimental site of Tibet, a region known to be very sensitive to anthropogenic perturbation and global warming. Our results showed that grazing changed microbial community functional structure, in addition to aboveground vegetation and soil geochemical properties. Further statistical tests showed that microbial community functional structures were closely correlated with environmental variables, and variations in microbial community functional structures were mainly controlled by aboveground vegetation, soil C/N ratio, and NH_4^+-N. In-depth examination of N cycling genes showed that abundances of N mineralization and nitrification genes were increased at grazed sites, but denitrification and N-reduction genes were decreased, suggesting that functional potentials of relevant bioprocesses were changed. Meanwhile, abundances of genes involved in methane cycling, C fixation, and degradation were decreased, which might be caused by vegetation removal and hence decrease in litter accumulation at grazed sites. In contrast, abundances of virulence, stress, and antibiotics resistance genes were increased because of the presence of livestock. In conclusion, these results indicated that soil microbial community functional structure was very sensitive to the impact of livestock grazing and revealed microbial functional potentials in regulating soil N and C cycling, supporting the necessity to include microbial components in evaluating the consequence of land-use and/or climate changes.

Keywords: climate change, gene diversity, microbial community, summer grazing, Tibetan alpine grassland

Received 18 May 2012 and accepted 11 October 2012

Introduction

Land use and climate change are projected to have a strong influence on biodiversity, with particular importance in the tropical or alpine regions (Sala *et al*., 2000). Livestock grazing is a global land-use activity with far-ranging societal and environmental impacts. It is the major biotic factor that influences pasture ecosystems, which span up to one quarter of the world's land area (Bokdam & Gleichman, 2000; FAO, 2008). The effects of grazing can be divided into three factors: vegetation removal, manure deposition, and trampling (Cingolani *et al*., 2003; Kohler *et al*., 2005). Vegetation removal changes the allocation of carbon (C) and nitrogen (N) between above- and belowground, and increases soil-extractable C in the rhizosphere (Holland & Detling, 1990; Guitian & Bardgett, 2000). Manure deposition accelerates N cycling by efficiently recycling nutrients through the animal excreta pathway (Kohler *et al*., 2005).

Correspondence: Yunfeng Yang, tel. +86-10-62784692, fax +86-10-62785687, e-mail: yangyf@tsinghua.edu.cn

Trampling compacts soil and hence decreases air permeability and hydraulic conductivity. Nevertheless, these factors intertwine, rendering it difficult to predict the net effect of grazing. For instance, N_2O emission is increased by urine deposition that stimulates N cycling (Yamulki *et al*., 1998; Wang *et al*., 2006b). As a result, substantial emission of greenhouse gases such as CH_4 and N_2O has been observed for pasture soil (Radl *et al*., 2007; Simek *et al*., 2007, 2009). However, it can be offset by reducing denitrification and microbial biomass, resulting in a recent finding that grazing decreases N_2O emission in grasslands of Inner Mongolia, China (Wolf *et al*., 2010).

Soil microbial community plays critical roles in regulating ecosystem function and soil biogeochemistry (Giller *et al*., 2004; Morin & McGrady-Steed, 2004). It mediates energy and material fluxes of ecosystems and alters soil biogeochemical properties (Chapin *et al*., 2000). Meanwhile, it is subjected to the influences of environmental changes at the levels of structure and activities (Bardgett *et al*., 2001). On the other hand, there is a growing number of studies to investigate how changes in microbial community affect stability and quality of ecosystem processes, as well as biodiversity and productivity of aboveground vegetation (Yeates *et al*., 1997; Wardle *et al*., 1999; Guitian & Bardgett, 2000; Clegg, 2006). Therefore, soil microbial community, in addition to plants and animals, must be considered in elucidating the mechanisms of ecosystem function and assessing the environmental impact of land-use changes.

Although microbes comprise much of the biodiversity on earth and are vital components in ecosystem functioning (Torsvik *et al*., 2002; Venter *et al*., 2004), there is still paucity of data to understand the interactions of microbial community members, the robustness and resilience of microbial community, and the response to environmental changes at the levels of both individual species and whole community (Huber *et al*., 2007; Allison & Martiny, 2008). One of the primary reasons stems from technical difficulties in studying microbial community. The recent development of high-throughput metagenomics technologies offers unprecedented opportunities for studying microbial community. The use of sequencing technologies has been successful in analyzing environmental microbiomes (Huber *et al*., 2007; Pointing *et al*., 2009; Dick & Tebo, 2010). Nevertheless, sequencing technologies are limited in that they are sensitive to random sampling errors, presence of contaminated DNA, and influence from the dominant organisms of microbial community (Zhou *et al*., 2011). GeoChip, a high-throughput, microarray-based metagenomics tool, is an excellent alternative (He *et al*., 2007, 2010b), despite that the reliability of GeoChip can be compounded by issues of probe specificity and coverage. Its most advanced version, GeoChip 4.0, contains probes to detect a staggering number of ~152,000 functional genes important to biogeochemistry, ecology, and environmental sciences.

In this study, we use GeoChip 4.0 to assess the impacts of livestock grazing on soil microbial community in Tibetan Plateau, which contains the largest grassland of the Eurasian continent (Zheng *et al*., 2000; Wang *et al*., 2010). Tibet is considered the third pole of the earth for its high altitude (Qiu, 2008). Its fragile ecosystem is sensitive to climate change and anthropogenic perturbation (Cao *et al*., 2004; Yang *et al*., 2006). It is estimated that more than 50 million sheep and 13.3 million domestic yaks (Yao *et al*., 2006), in addition to native grazers, graze on pastoral lands of Tibet, imposing a marked pressure on the alpine grassland. Concurrently with rising temperatures of the region (Duan *et al*., 2006), it is crucial to evaluate the impact of livestock grazing in Tibetan grassland.

Although grazing effects on the aboveground vegetation and soil physicochemical properties of Tibetan alpine grassland have been documented in recent years (Klein *et al*., 2004, 2007; Wang *et al*., 2010), few studies consider grazing effects on soil microbes. To address it, we initiated a study to investigate: (i) whether ungrazed and grazed sites differed in microbial community functional structure; (ii) whether the differences in microbial community structure were controlled by a few environmental factors; (iii) whether and how functional genes were changed by grazing; and (iv) whether changes of key functional genes aligned with microbe-mediated functional processes.

Materials and methods

Site and sampling

The study was conducted at Haibei Alpine Meadow Ecosystem Research Station, which is situated at latitude 37.37°N, longitude 101.12 E of Tibet. The mean annual air temperature and precipitation is −1.7 °C and 560 mm, respectively (Zhao *et al*., 2006). The soil is silty clay loam of Mat Cry-gelic Cambisols with pH of 7.3 and 7.4, bulk density of 0.75 and 1.11 g cm^{-3} at depths of 10 and 20 cm, respectively. The aboveground vegetation is dominated by C_3 herbal species such as *Kobresia humilis* and *Potentilla nivea* (Zhao *et al*., 2006).

At elevations of 3600 and 3800 m, the grassland has been subjected to free livestock grazing in summer (from April to September) for decades. To allow for comparison between ungrazed and grazed sites, three sites of 1 × 1 m size were fenced at each elevation in May 2006 to exclude livestock grazing. Three adjacent sites of the same size continued to be open for grazing.

Soil samples of ungrazed and grazed sites were collected in August, 2009, and used for GeoChip analysis. At each site, five soil cores at depth of 0–20 cm and diameter of 1.5 cm were collected on a grid basis and composited. The soil samples were

kept on ice, transported back to the laboratory, and sieved through 2 mm mesh to remove most of visible roots and stones. Then soil samples were stored at −80 °C until DNA extraction.

Soil and vegetation property measurements

Soil temperature was measured using type-K thermocouples (Campbell Scientific, Logan, UT, USA) coupled to a CR1000 datalogger installed at the depths of 5 and 10 cm at the time of sampling, respectively. The average soil moisture at the 0–10 cm depth was measured using a frequency domain reflectometer Model Diviner-2000 (Sentek Pty Ltd., Stepney, Australia) through a tube inserted in the soil down to the depth of 10 cm at the time of sampling.

Other soil properties were measured at the depth of 0–10 and 10–20 cm, respectively. Total organic C (TOC) was measured by a TOC-5000A analyzer (Shimadzu Corp., Kyoto, Japan). Total N of soil was measured by a Vario EL III Elemental Analyzer (Elementar, Hanau, Germany) as previously described (Wang *et al*., 2009). To measure NH_4^+-N and NO_3^--N, 10 g dry weight of soil samples was suspended in 50 ml of 2 M KCL solution. After shaking at room temperature for 1 h and subsequent standing for 30 min, the supernatant was filtered through a filter paper of 30–50 μm pore size. NH_4^+-N and NO_3^--N were analyzed by a FIAstar 5000 Analyzer (FOSS, Hillerd, Danmark). The measurements of greenhouse gases (CO_2, N_2O, and CH_4) were conducted as described previously (Wang *et al*., 2009). To measure vegetation variables, a quadrat in the site was selected. The vegetation species, density, abundance, and average height were recorded according to an established protocol (Klein *et al*., 2007). Then vegetation were mowed and immediately weighted for biomass data. Vegetation diversity was measured using Shannon-Weaver index.

DNA extraction, purification, and quantitation

DNA was extracted from 0.5 g soil using FastDNA spin kit for soil (MP Biomedical, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was further purified by precipitating with 2.5 vol. of 100% ice-cold ethanol and 0.1 vol. of 3 M NaOAc (pH 5.2). After incubation overnight at −20 °C, the mixture was centrifuged for 30 min at 13 000 × *g*. The supernatant was decanted and DNA pellet was washed with 1 ml ice-cold 70% ethanol. After centrifugation for 10 min at 13 000 × *g*, the supernatant was carefully removed and DNA pellet was air dried for 30 min. Nuclease-free water was added to dissolve DNA. DNA was repurified using column-based method (Zhou *et al*., 1996) and DNA quality was assessed by the ratios of $A_{260/280}$ and $A_{260/230}$ using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and final soil DNA concentrations were quantified with PicoGreen (Ahn *et al*., 1996) using a FLUOstar Optima (BMG Labtech, Jena, Germany).

GeoChip hybridization and scanning

GeoChip 4.0 (Hazen *et al*., 2010; Lu *et al*., 2012) was used for analyzing DNA samples. The experiments were performed essentially as described previously (Lu *et al*., 2012). In brief, DNA was labeled with the fluorescent dye Cy-5 using a random priming method and then purified with the QIA quick purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After measuring dye incorporation on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), DNA was dried in a SpeedVac (ThermoSavant, Milford, MA, USA) at 45 °C for 45 min. Subsequently, labeled DNA was resuspended in 120 μl hybridization solution containing 40% formamide, 3 × SSC, 10 μg of unlabeled herring sperm DNA (Promega, Madison, WI, USA), and 0.1% SDS, and hybridizations were performed with a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA) according to the manufacturer's recommended method. After washing and drying, microarray was scanned by a NimbleGen MS200 scanner (Roche, Madison, WI, USA) at 633 nm using a laser power of 100% and a photomultiplier tube (PMT) gain of 75%. Signal intensities were subsequently quantified.

Data analyses

Raw data were analyzed using a data analysis pipeline as described previously (He *et al*., 2010b). In brief, the following steps were performed: (i) spots flagged or with a signal to noise ratio (SNR) less than 2.0 were removed as poor-quality spots; (ii) after removing poor spots, normalized intensity of each spot was calculated by dividing the signal intensity of each spot by the total intensity of the microarray and then multiplying it with a constant value of 155772337, which is the average signal intensity of all GeoChip data; (iii) natural logarithmic transformation was applied; and (iv) a minimum of two valid values of three biological replicates, that is, samples from the same elevation and treatment, was required for each gene.

Principal Component Analysis (PCA) was used to determine overall functional changes in microbial communities. For the dissimilarity test of adonis, we used Bray-Curtis and Srensen distances to calculate the dissimilarity distance matrices from GeoChip hybridization data, which are based on dissimilarities among samples and their rank order in compliance to adonis method to calculate test statistics, and the Monte Carlo permutation was used to test the significance of statistics. The similarity test between GeoChip and environmental variables was conducted by calculating Euclid distances among samples and then plotted to calculate Pearson correlation.

We also evaluated possible linkages between the functional gene structure of microbial communities (based on GeoChip data) and soil and vegetation variables to elucidate interrelationships among vegetation, soil variables, and microbial community. First, all of soil or vegetation variables were included in Mantel test and the CCA modeling. They were virtually meaningless due to the noise and redundancy among those variables for soil or vegetation. Then, the BioEnv procedure (Clarke & Ainsworth, 1993) and variance inflation factor (VIF) were used for step-wise removal of redundant variables in CCA modeling, resulting in selection

of three soil variables (NH_4^+-N, NO_3^--N, and C/N ratio) and three vegetation variables (vegetation biomass, Shannon diversity, and the count of species). For Mantel test, Bray-Curtis coefficient and Euclidean distance were used to construct dissimilarity matrices of communities and environmental variables, respectively. All of the analyses were performed by functions in the vegan package (v.1.15-1) in R v. 2.8.1 (Dixon, 2003).

Results

Effects of grazing on soil microbial community structure

Soils of grazed (g) and ungrazed (c) sites at the 3600 m and 3800 m elevation were sampled. DNA was extracted from the samples and analyzed by GeoChip 4.0. A total of 51 945 genes were detected in this experiment. The dissimilarity test of adonis indicated that grazing imposed changes in GeoChip 4.0 and environmental variables to both 3600 and 3800 m sites, albeit it was marginally significant for 3800 m sites (Table S1). Hierarchical clustering analysis indicated that samples were well grouped according to grazing or elevation (Fig. S1), demonstrating that site differences at the same elevation were minor compared with differences in grazing or elevation. To confirm these observations, PCA was conducted using the whole GeoChip dataset. Samples were grouped by grazing or elevation, and samples from grazed sites were well separated from the ungrazed ones (Fig. 1).

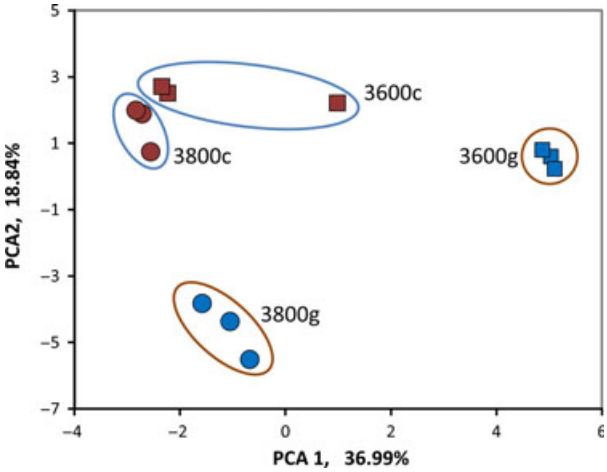

Fig. 1 Principal Component Analysis of GeoChip data. Circles and triangles represent ungrazing and grazing samples, respectively. The values of Axis 1 and 2 are percentages of total variations that can be attributed to the corresponding axis.

Examination of GeoChip results indicated that alpha diversity, represented as Shannon and Simpson diversities, was significantly ($P < 0.03$) smaller at grazed sites than those at ungrazed sites (Table 1). The gene richness was also significantly ($P < 0.03$) different between ungrazed and grazed samples. These results indicated that grazing reduced microbial functional diversity at these sites.

Table 1 Diversity indices of functional genes

Gene categories	Indices	3600c	3600g	*P* value	3800c	3800g	*P* value
All GeoChip genes	Richness index*	37612	30430	**0.027**§	38545	30771	**0.011**
	Shannon index (H)†	10.53	10.32	**0.025**	10.56	10.33	**0.015**
	Simpson's diversity index (1/D)‡	37307.78	30230.26	**0.026**	38260.68	30556.36	**0.010**
	Pielou (Shannon) evenness (J)	0.9996	0.9997	0.309	0.9997	0.9997	0.787
	Simpson evenness	0.9920	0.9934	0.274	0.9926	0.9930	0.653
C cycling genes	Richness Index*	4907	3968	**0.028**	5044	4033	**0.013**
	Shannon index (H)†	8.49	8.28	**0.026**	8.52	8.30	**0.018**
	Simpson's diversity index (1/D)‡	4867.41	3942.81	**0.027**	5007.62	4006.07	**0.012**
	Pielou (Shannon) evenness (J)	0.9995	0.9996	0.233	0.9996	0.9996	0.827
	Simpson evenness	0.9921	0.9937	0.201	0.9929	0.9933	0.671
N cycling genes	Richness index*	3492	2863	**0.030**	3591	2792	**0.009**
	Shannon index (H)†	8.15	7.96	**0.028**	8.18	7.93	**0.013**
	Simpson's diversity index (1/D)‡	3465.46	2845.36	**0.029**	3566.09	2773.27	**0.009**
	Pielou (Shannon) evenness (J)	0.9995	0.9996	0.255	0.9996	0.9996	0.987
	Simpson evenness	0.9925	0.9940	0.223	0.9931	0.9933	0.780

*Detected gene numbers.
†Shannon index, higher number represents higher diversity.
‡Reciprocal of Simpson's index, higher number represents higher diversity.
§Values of significance at $P < 0.05$ are shown in bold.

Relationship between microbial community structure and environmental variables

GeoChip results indicated that livestock grazing changed both microbial community structure and environmental variables. As grazing effects on microbial community were possibly mediated by aboveground vegetation and soil geochemical variables, we explored whether there existed a relationship between microbial community structure and environmental variables (Table S2). The results unveiled a strong relationship between microbial community structure and environmental variables (Fig. 2a). Therefore, CCA was performed to reveal major environmental variables shaping microbial community structure. To eliminate variables that depend on other measured variables, automatic forward selection and variance inflation factors (VIF) were used. The significance was based on 999 Monte Carlo permutations. The top seven variables (C/N ratio, NH_4^+-N, NO_3^--N, elevation, total vegetation biomass, vegetation diversity, and species numbers) were then selected as inputs for CCA. As shown in Fig. 2b, both soil variables (C/N ratio and NH_4^+-N) and vegetation variables (vegetation biomass and diversity) showed a significant ($P < 0.05$) relationship with microbial community structure, which was also supported by Mantel test at $P < 0.05$ (Table 2). Furthermore, C/N ratio and NH_4^+-N were major factors linking to the microbial community structure of 3600 g samples, suggesting that grazing-induced N cycling activity had a strong relationship with the microbial community of 3600 g sites. Meanwhile, vegetation removal by grazing, indicated by vegetation biomass data (Table S2), showed distinct impact on microbial communities and differentiated the grazed samples from the ungrazed.

Variance partition analysis was subsequently performed to dissect the contribution of elevation, vegetation, and soil variables to the microbial community structure. A total of 81.7% of the community variations could be explained by these selected variables (Fig. 2c). Elevation, vegetation, and soil variables contributed to 7.7%, 25.5%, and 22.7% of the total variance, respectively. Moreover, vegetation and soil variables together contributed extra 11.1% of the total variance, indicating that there were considerable interactions between soil and vegetation variables.

N cycling genes

Significant relationship between microbial community structure and C/N ratio and NH_4^+-N provoked us to investigate N cycling genes. As urine deposition by livestock provided a unique N source to grazed sites

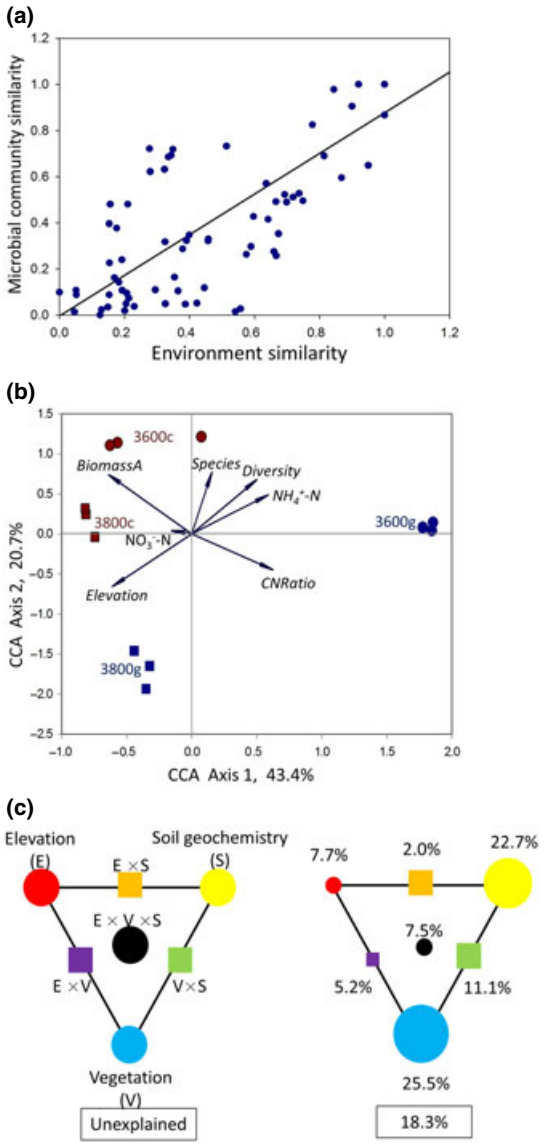

Fig. 2 (a) The similarity test between GeoChip and environmental variables. Euclid distances among samples were calculated and plotted. (b) Canonical correspondence analysis (CCA) using all detected genes (symbols) of GeoChip data and seven selected environmental variables (arrows). The values of Axis 1 and 2 are percentages that the corresponding axis can explain. (c) Variation partitioning analysis that partitions relative influence of environmental variables on microbial community structure. Environmental variables are divided into groups of soil (C/N ratio, NO_3^--N, and NH_4^+-N), vegetation (biomass, species, and diversity), and elevation. The circles represent individual variable group by partitioning out the effects of other groups. The geometric areas of the circles are proportional to the respective percentages of effects as indicated by the numbers. The squares between the circles represent the joint effect of the circles on both sides of the square. The portion unexplained by any of tested variable groups is shown in the square at the bottom of the figure. *P* values were determined to be 0.05 by partial CCA.

Table 2 Correlation between functional genes of microbial community (all detected genes and *nifH*) and environmental variables as shown by Mantel tests

	Soil†	Vegetation‡	Soil T§	Air T	Moisture	NO_3^--N
GeoChip	0.359**	0.420***	0.366**	0.366***	0.631***	0.077
nifH	0.372**	0.428***	0.390**	0.390***	0.093	0.085
	NH_4^+-N	TOC	CN ratio	Released CH_4	Released CO_2	Released N_2O
GeoChip	0.215*	0.492***	0.301**	−0.054	0.295**	−0.106
nifH	0.239**	0.529***	0.297**	−0.043	0.349**	−0.088
	TN	Soil inorganic N	Species	Biomass	Diversity	Elevation
GeoChip	0.314**	0.151	0.026	0.431***	0.309**	0.366**
nifH	0.351**	0.151	0.038	0.408***	0.324**	0.390***

†Soil variables: Soil T, Moisture, NO_3^--N, NH_4^+-N, TOC, TN, CH_4, CO_2, and N_2O.
‡Vegetation variables: vegetation biomass, species, and diversity.
§T, temperature; TOC, Total organic carbon; TN, Total N; CNRatio, the ratio of TOC and TN; Species, Species number of vegetation; Biomass, Aboveground vegetation biomass; Diversity, Aboveground vegetation diversity.
***$P < 0.01$, **$P < 0.05$, *$P < 0.1$.

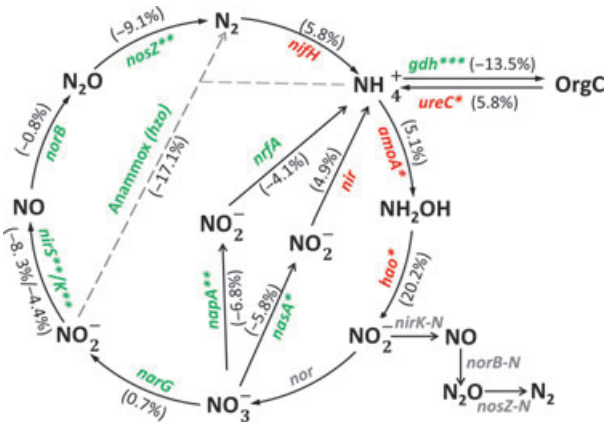

Fig. 3 Changes in N cycling gene abundances at grazed sites. The percentages in the bracket indicate fold changes in the normalized total intensity of the functional gene family between grazed and ungrazed sites. Red and green represent the increase and decrease in gene abundance by grazing, respectively. The gray-colored genes are not targeted by GeoChip. ***$P < 0.01$, **$P < 0.05$, *$P < 0.1$.

(Roux *et al*., 2003), we examined *ureC* and *gdh* functioning to convert urea into ammonia and *vice versa*, respectively. GeoChip data showed that the abundance of *gdh* was decreased whereas that of *ureC* was increased (Fig. 3). Thereby, the combined effect of *gdh* and *ureC* appeared to shift the balance of urea metabolism toward urea ammonification and hence increased the N mineralization potential. The most increased *ureC* genes included those derived from *Alteromonas macleodii*, *Dechloromonas aromatica*, *Deinococcus radiodurans*, *Helicobacter acinonychis str. Sheeba*, *Loktanella vestfoldensis*, *Mycobacterium tuberculosis*, *Nitrosospira sp.*, *Octadecabacter antarcticus*, *Pyrenophora tritici-repentis*, *Rhodococcus sp.*, *Staphylococcus carnosus*, *Variovorax paradoxus*, *Vibrionales bacterium*, and unidentified bacteria (Fig. 4), suggesting that these species could be selectively stimulated by grazing. In addition, there were genes derived from a number of *Nitrosospira* species, which were classified to be ammonia-oxidizing *β*-proteobacteria. This was consistent with the important role of this group in N cycling and wide distribution in soil environments (Head *et al*., 1993).

GeoChip contained probes to detect 16 key functional N gene families involved in nitrification, denitrification, N fixation, dissimilatory N reduction, assimilatory N reduction, or anammox. Our results indicated that gene abundances of denitrification, dissimilatory, and assimilatory N reduction were decreased at grazed sites, whereas total abundances of 659 detected nitrification genes were increased (Fig. 3). For nitrification, *amoA* genes derived from ammonia-oxidizing archea (AOA) and ammonia-oxidizing bacteria (AOB) were increased. Notably, AOA/AOB ratios were similar at grazed and ungrazed sites (data not shown), suggesting that grazing increased the abundance of AOA and AOB to similar extent. The increased AOB included a wide range of phyla such as *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria*. Therefore, changes in *amoA* genes appeared not to be inclined for certain phyla. In addition, we examined the correlation between amoA genes and N_2O emission as nitrification was a driving force of N_2O emission. A number of genes derived from alphaproteobacteria were identified to be significantly ($P < 0.01$) correlated with N_2O emission, including those derived from *Mesorhizobium loti*, *Oceanicola batsensis*, and *Octadecabacter antarcticus* species.

For denitrification, abundances of *nirS*, *nirK*, and *nosZ* genes were significantly decreased, whereas that of *norB* remained unchanged. Similar to the observation of phyla distribution for *amoA* genes, changes in

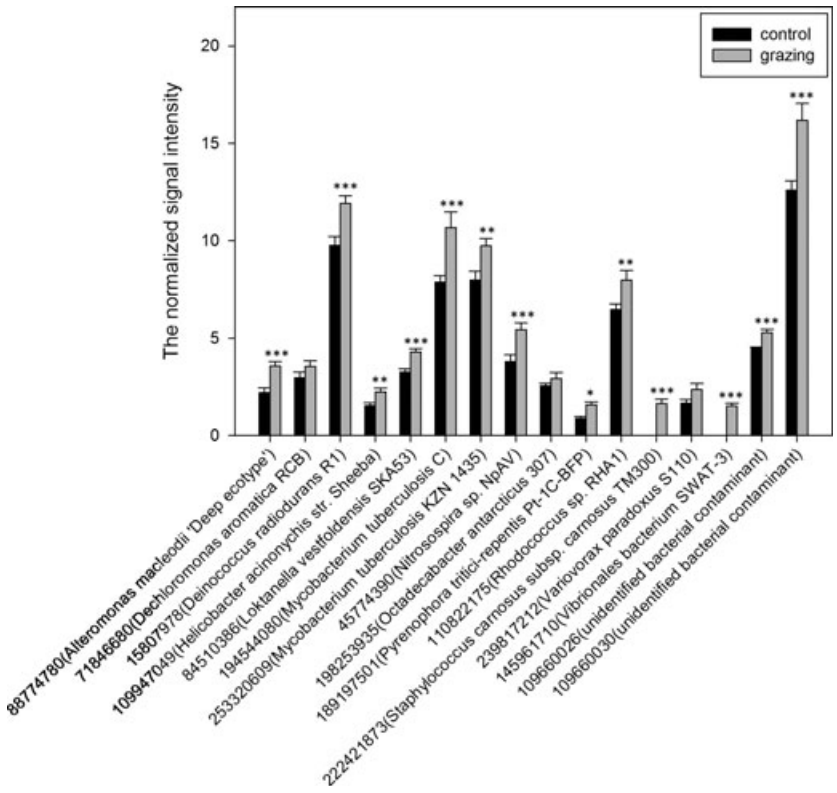

Fig. 4 The normalized signal intensity of the most increased *ureC*. Gene number is the protein ID number for each gene as listed in the GenBank database. All data are presented as mean ± SE. ***$P < 0.01$, **$P < 0.05$, *$P < 0.1$ as examined by *t*-test.

denitrification genes occurred in all of the major branches of microbial phyla present on GeoChip. Together, these results suggested that regulation of these bioprocesses did not target specific bacteria. Meanwhile, Mantel tests indicated that *nifH*, a key gene of N fixation, was significantly correlated with NH_4^+-N, C/N ratio, total N, total organic C, vegetation biomass, and diversity at $P < 0.05$ (Table 2). The top 10 upregulated genes were derived from *Anabaena variabilis*, *Methanococcus aeolicus*, *Paenibacillus massiliensis*, and several uncultured bacteria (data now shown).

C cycling genes

The abundances of most C fixation and degradation genes were either reduced or unchanged by grazing (Fig. 5a,b), suggesting that the microbe-mediated C cycling potential was inhibited. In accordance, CO_2 emission and soil TOC were decreased and increased, respectively (Table S2). Thus, grazing seemed to promote net C storage at our sites.

Three of four major C fixation gene families targeted by GeoChip were decreased, all of which were derived from bacteria except a few (*rubisco* derived from uncultured methanogenic archaeon, and *pcc* derived from *Haloarcula marismortui* and *Hyperthermus butylicus*). Meanwhile, substantial variations within C degradation genes were notable. A multitude of genes seemed to be sensitive to grazing as they became undetectable at grazed sites, which included *amyA* genes derived from *Psychromonas ingrahamii 37*, *Cellvibrio japonicus Ueda107*, *Thermoanaerobacter mathranii subsp. mathranii str. A3*, *Vibrio orientalis CIP102891*, and *Erythrobacter litoralis HTCC2594*. In contrast, there were also many genes detected in high abundance across all samples, including acetylglucosaminidase genes derived from *Gluconacetobacter diazotrophicus PAl5* and *Streptomyces roseosporus NRRL11379*, arabinofuranosidase gene derived from *Aspergillus terreus NIH2624*, and lignin peroxidase gene derived from *Pseudotrametes gibbosa*. There were also exceptions of genes that were significantly increased at grazed sites (Fig. 5b). Among them, there were amylopullulanase genes, which functioned in starch utilization.

Three key functional genes related to CH_4 emission were analyzed by GeoChip. *mcrA* encoded an enzyme that was considered as a diagnostic indicator of methanogenesis (Luton *et al.*, 2002), whereas *pmoA* and *mmoX* encoded for the two methane monooxygenases. Response ratios showed that whereas the abundance of *pmoA* remained unchanged, those of *mcrA* and *mmoX* were decreased at grazed sites (Fig. 5c). Twelve *mcrA*

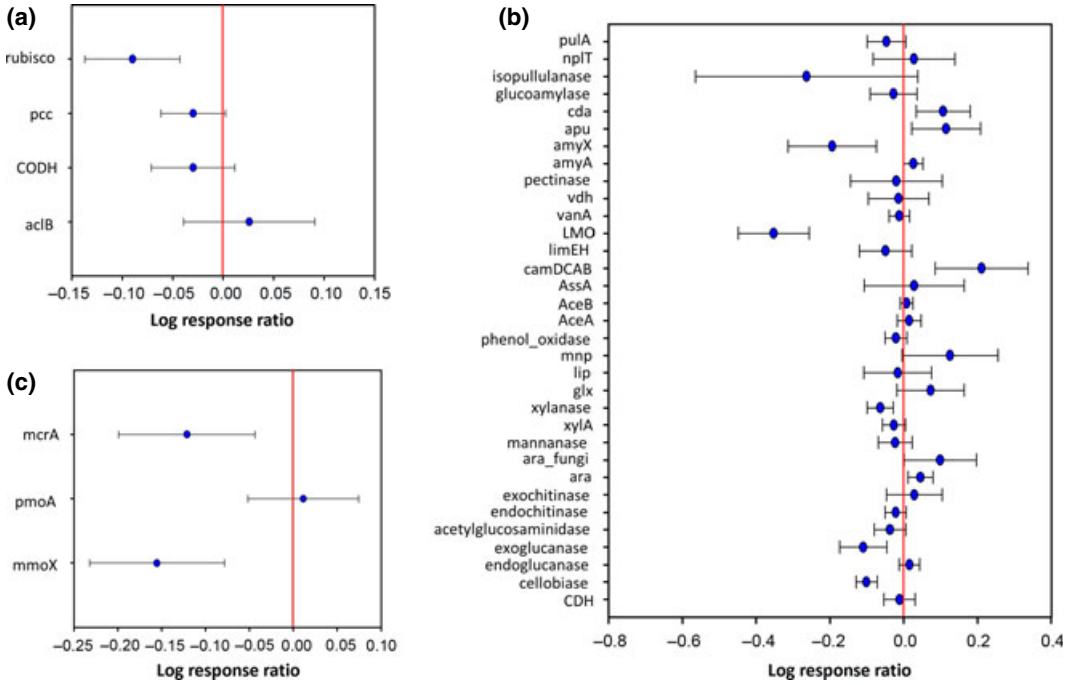

Fig. 5 Response ratios to compare gene abundance under ungrazing and grazing conditions for (a) C fixation; (b) C degradation; and (c) CH_4 cycling genes at the confidence level of 0.90.

genes, which were mainly derived from uncultured methanogenic archaeon except for *Methanosarcina mazei Go1*, appeared to be sensitive to grazing as they were reduced at both grazed sites (Fig. S2).

Virulence, stress, and antibiotics resistance genes

Response ratio analysis showed that gene categories of virulence and stress were marginally significantly ($P < 0.1$) more abundant at grazed sites (Fig. 6). The category of antibiotics resistance was also notably more abundant, albeit insignificant at the confidence level of $P < 0.1$.

A multitude of increased virulence genes were derived from *Clostridia*, which was prevalent in animal gut (Hess *et al.*, 2011). The most highly increased genes included those derived from mesophilic Clostridia of *C. phytofermentans*, *C. acetobutylicum*, *C. botulinum*,

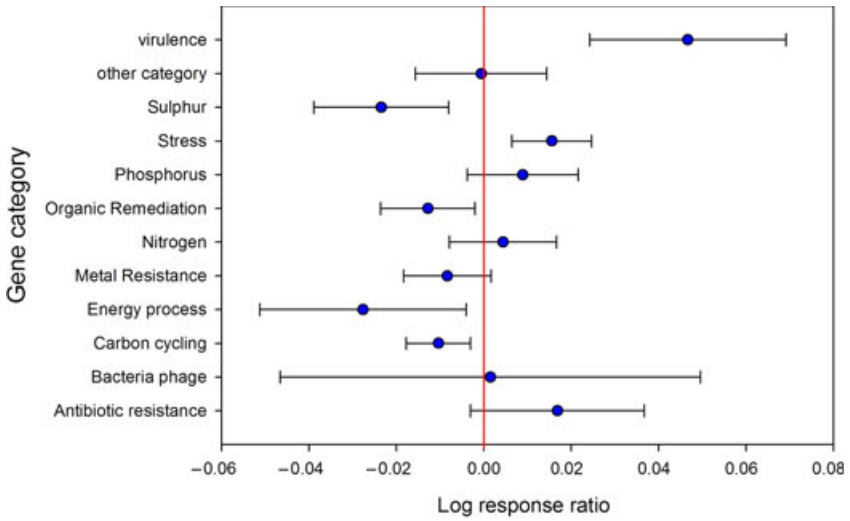

Fig. 6 Response ratios to compare gene abundance at ungrazed and grazed sites for all of the gene categories detected by GeoChip at the confidence level of 0.90. Total abundances of detected individual genes in the same gene categories were used for calculating response ratios.

C. perfringens, and *C. tetani*. *C. phytofermentans* and *C. acetobutylicum* were known for their efficiency in cellulose degradation or butanogenesis (Mermelstein *et al*., 1992; Liu *et al*., 2010), whereas others had close relatives that were pathogenic to animal health (Sebaihia *et al*., 2007).

The significantly ($P < 0.1$) induced stress genes were responsive to a variety of stress types, including *sigma 24* (heat-induced transcription initiation), *sigma 70* (general transcription initiation), *pstB* (phosphate transporter subunit), *narL* and *narH* (respiratory nitrate reductase subunits), *cydB* (cytochrome d ubiquinol oxidase subunit II), *arcB* (aerobic respiration control sensor protein), *opuE* (sodium/proline symporter), *tnrA* (N sensing transcriptional regulator), *hrcA* (heat-inducible transcription repressor), *bglH* (glucoside-specific outer membrane porin), and *desK* (two-component sensor histidine kinase of cold stock). In addition, a variety of bacteria were subjected to the influence of stress as indicated by the wide range of bacterial phyla from which the stress genes were derived (data not shown). For antibiotics genes, the most increased genes were *van, tet,* and *B lactamase* conferring resistance to vancomycin, tetracycline, and beta-lactam antibiotics, respectively. Similar to the observation of stress genes, the increased antibiotics genes were derived from a variety of bacterial phyla such as *Actinobacteria*, *Firmicutes, Verrucomicrobia,* and *Proteobacteria*.

Discussion

Livestock grazing is a major land use with extensive economic and environmental consequences. Accumulating evidences have linked livestock grazing to greenhouse gas emission and thus climate changes because of grazing effects on vegetation litter, soil aeration, and manure deposition (Yamulki *et al*., 1998; Wang *et al*., 2006b; Radl *et al*., 2007; Simek *et al*., 2007, 2009; Wolf *et al*., 2010). As microbes are crucial for ecosystem function as important engines in driving biogeochemical cycles (Grime, 1997; Hooper & Vitousek, 1997; Duffy *et al*., 2001; Thompson *et al*., 2005; Falkowski *et al*., 2008), it is necessary to understand the molecular mechanisms of microbe-mediated processes. To address it, we adopted GeoChip 4.0 to dissect microbial community functional structure. Although GeoChip is limited in representing microbial species identity and the breadth of *in situ* microbial community, it can overcome several major problems of sequencing technology (Zhou *et al*., 2011) and thus has been successfully used in dissecting functional potentials of microbial communities (Van Nostrand *et al*., 2009; He *et al*., 2010a; Lu *et al*., 2012).

Our results showed that the structure of soil microbial community clearly underwent changes, which was in consistency with changes in soil and vegetation variables. Although this observation was not surprising in light of previous studies dedicated to grazing effects of microbial community structure, most of such studies addressed grazing effects at the species level (Dowd *et al*., 2008; Jesus *et al*., 2009) or focused on single functional process (Wright *et al*., 2007; Le Roux *et al*., 2008). This is limited because knowing the species is insufficient to infer its roles in regulating material and energy flow of the ecosystem (Chapin *et al*., 2000), and ignoring the interaction among functional processes resulted in inaccurate estimation of outcomes of the ecosystem function. Therefore, it is crucial to focus on a more complete set of microbial functional genes to achieve a better understanding of microbe-mediated processes.

Microbial biomass and/or diversity can be increased, decreased, or unchanged, depending on grazing regimes and intensities (Bardgett & Wardle, 2003; Harrison & Bardgett, 2004; Mills & Adl, 2006; Wang *et al*., 2006a). Therefore, microbial communities do not respond in a uniform way. Also, it seems that aboveground vegetation plays a role in determining microbial community as vegetation can select soil microbes to be associated with (de Goede *et al*., 2002; Bardgett & Wardle, 2003; Ebersberger *et al*., 2004; Colon-Carmona *et al*., 2009). The grazing effect on aboveground vegetation is apparent as indicated by the reduction in vegetation biomass (Table S2). In addition, grazing decreased the proportion of herbaceous vegetation, which were abundant in cellulose and lignin components, and increased the proportion of Artemisia, which were abundant in N content (Bates *et al*., 2009). Thus, grazing can change vegetation composition as well. Accordingly, our Mantel tests showed significant correlations between microbial community functional structure and vegetation biomass ($r = 0.431$, $P < 0.008$) and diversity ($r = 0.309$, $P < 0.02$), respectively (Table 2). Therefore, changes in aboveground vegetation have a clear linkage to those of belowground microbial community. In addition, it was noted that changes in aboveground vegetation corresponded to the decrease in C degradation gene abundance (e.g., *cellobiase* and *exoglucanase*) (Fig. 5c) and the increase in N mineralization and nitrification gene abundance (*ureC* and *amoA*) (Fig. 3).

Also, soil geochemical variables appeared to play an essential role in shaping microbial community. For example, patches of bare soil caused by grazing could lead to loss of soil organic matter by wind erosion (Hoffmann *et al*., 2008; Kolbl *et al*., 2011), which in turn affects microbial biomass and activity (Holt, 1997). The essential role of soil variables in shaping microbial community was apparent, as indicated by a strong positive correlation between microbial functional structure and soil organic C ($r = 0.492$, $P < 0.003$), total N

($r = 0.314$, $P < 0.05$), and soil moisture ($r = 0.631$, $P < 0.01$) (Table 2).

Nevertheless, correlations between microbial community and environmental factors must be interpreted with caution because it is often difficult to firmly establish a relationship between microbial community and soil nutrient cycling (Bardgett, 2005). One of possible explanations is that biotic factors only control part of ecosystem processes. It is also likely that functional gene abundance, which represents genetic potential, is not necessarily in accordance with microbial activity, which is more pertinent to ecosystem processes. Furthermore, changes at the gene or species levels may not be scaled up to the level of ecosystem processes. Bearing in mind these caveats, it was still striking to note the remarkable correlation between microbial community functional structure and environmental variables (Fig. 2a), which was further supported by the observation that the portion of the total unexplained variation (Fig. 2c) was small compared with several recent GeoChip studies (Van Nostrand *et al*., 2009; Liang *et al*., 2011; Lu *et al*., 2012). This indicated that the identified environmental variables were major factors influencing the microbial community structure. As a few environmental variables were sufficient in explaining a majority of variations in microbial community functional structure, it seemed to suggest that stochastic factors played a relative minor role in shaping microbial community, as one would expect for harsh environments of Tibet as the third pole of the earth.

Livestock grazing has important effects on soil N cycling (Ruess & Mcnaughton, 1987), which are influenced by animal excreta N return, compaction of soil bulk density through animal trampling, and changes in modified N input rates and quality of litter accumulation, to name a few (Roux *et al*., 2003; Le Roux *et al*., 2008). Consequently, soil N cycling could vary by grazing intensity/regimes, grassland types, and habitat conditions. Increased nitrification was reported previously in grasslands and prairies (Groffman *et al*., 1993; Tracy & Frank, 1998), which was supported by modeling (Seagle & McNaughton, 1993). Denitrification can also be stimulated following urine deposition (Monaghan & Barraclough, 1993) or defoliation events (Roux *et al*., 2003), but can also remain unchanged or even be repressed under certain conditions such as overgrazing (Groffman *et al*., 1993). Our results showed that livestock grazing increased soil N mineralization potential (Fig. 3), which was in consistency with general observation (Ruess & Mcnaughton, 1987; Tracy & Frank, 1998). The increase in nitrification and decrease in denitrification were interesting, which had influence on the balance between NH_4^+-N and NO_3^--N and consequently N conservation of the sites. This shift could also affect N_2O emission.

Vegetation removal by grazing caused less litter input to soils. Accordingly, abundances of most C fixation, C degradation, and CH_4 cycling genes were significantly lower in grazing samples than in ungrazing samples (Fig. 5), suggesting that microbe-mediated C cycling was reduced. Accordingly, CO_2 emission was significantly reduced at 3800 m grazed sites (Table S2), whereas CH_4 emission was not altered. This finding raised an intriguing benefit of grazing in mitigating greenhouse gas emission, which was in alignment with the recent finding that grazing inhibited N_2O emission in the grasslands of Inner Mongolia, China (Wolf *et al*., 2010). Nonetheless, it is apparent that soil C retention and greenhouse gas emission are strongly influenced by grazing intensity and regime, thus further study is necessary to analyze their effects. Notably, correlation was observed between greenhouse gas fluxes and microbial activities (unpublished data), thus *in situ* microbial community might play an active role in mediating greenhouse gas fluxes on these sites. In addition, in consistency with previous studies in upland grasslands (Hütsch *et al*., 1994; Castaldi *et al*., 2007), Tibetan alpine grassland may have acted as a source for carbon dioxide rather than a sink for CH_4.

The increase in virulence, stress, and antibiotics resistance gene abundance by grazing is not surprising. The virulence genes are required for bacteria to colonize within the digestion system of animal hosts, which could be released from livestock dung (McAllister & Topp, 2012) and thus result in the increase in virulence genes at grazed sites. The increase in stress genes could be explained by their functional roles in bacteria's coping with the environmental changes stemmed from grazing or during bacterial infection of animal hosts. In addition, modern pasturing has widely used antibiotics to control animal disease (Kemper, 2008), which could be reflected in the upshift of antibiotics resistance genes.

In conclusion, we examined grazing effect on soil microbial community by focusing on functional genes. GeoChip results showed that grazing altered the structure of microbial community. Moreover, changes in microbial community were largely attributed to vegetation and soil variables. The increase in N mineralization and nitrification and the decrease in denitrification indicated that grazing altered microbe-mediated N cycling. The decrease in key genes involved in CH_4 and N_2O cycling sheds light on the microbial functional potential in mitigating greenhouse gas emission. Although it remains unclear whether these conclusions can be generalized to other alpine grasslands as this study has been conducted in one location with limited

sampling size, it represents an important step forward to understand the microbial functional potentials changed by livestock grazing.

Acknowledgements

The authors wish to thank Haibei Research Station staff for sampling assistance, and the anonymous reviewers for constructive comments. This research was supported by grants to Yunfeng Yang from National Science Foundation of China (41171201) and State Key Joint Laboratory of Environment Simulation and Pollution Control (11Z03ESPCT), to Shiping Wang from the National Basic Research Program (2010CB833502), to Jizhong Zhou from United States Department of Energy, Biological Systems Research on the Role of Microbial Communities in C Cycling Program (DE-SC0004601), and Oklahoma Bioenergy Center (OBC). The GeoChips and associated computational pipelines used in this study were supported by ENIGMA-Ecosystems and Networks Integrated with Genes and Molecular Assemblies through the Office of Science, Office of Biological and Environmental Research, of the U. S. Department of Energy under Contract No. DE-AC02-05CH11231, and by the United States Department of Agriculture (Project 2007-35319-18305) through NSF-USDA Microbial Observatories Program.

References

Ahn SJ, Costa J, Emanuel JR (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Research*, **24**, 2623–2625.

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11512–11519.

Bardgett RD (2005) *The Biology of Soil, a Community and Ecosystem Approach*. Oxford University Press, New York.

Bardgett RD, Wardle DA (2003) Herbivore-mediated linkages between aboveground and belowground communities. *Ecology*, **84**, 2258–2268.

Bardgett RD, Jones AC, Jones DL, Kemmitt SJ, Cook R, Hobbs PJ (2001) Soil microbial community patterns related to the history and intensity of grazing in sub-montane ecosystems. *Soil Biology & Biochemistry*, **33**, 1653–1664.

Bates JD, Rhodes EC, Davies KW, Sharp R (2009) Postfire succession in big sagebrush steppe with livestock grazing. *Rangeland Ecology and Management*, **62**, 98–110.

Bokdam J, Gleichman JM (2000) Effects of grazing by free-ranging cattle on vegetation dynamics in a ntinental north-west European heathland. *Journal of Applied Ecology*, **37**, 415–431.

Cao G, Tang Y, Mo W, Wang Y, Li Y, Zhao X (2004) Grazing intensity alters soil respiration in an alpine meadow on the Tibetan plateau. *Soil Biology and Biochemistry*, **36**, 237–243.

Castaldi S, Costantini M, Cenciarelli P, Ciccioli P, Valentini R (2007) The methane sink associated to soils of natural and agricultural ecosystems in Italy. *Chemosphere*, **66**, 723–729.

Chapin FS 3rd, Zavaleta ES, Eviner VT *et al.* (2000) Consequences of changing biodiversity. *Nature*, **405**, 234–242.

Cingolani AM, Cabido MR, Renison D, Solis VN (2003) Combined effects of environment and grazing on vegetation structure in Argentine granite grasslands. *Journal of Vegetation Science*, **14**, 223–232.

Clarke KR, Ainsworth M (1993) A Method of linking multivariate community structure to environmental variables. *Marine Ecology Progress Series*, **92**, 205–219.

Clegg CD (2006) Impact of cattle grazing and inorganic fertiliser additions to managed grasslands on the microbial community composition of soils. *Applied Soil Ecology*, **31**, 73–82.

Colon-Carmona A, Micallef SA, Shiaris MP (2009) Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates. *Journal of Experimental Botany*, **60**, 1729–1742.

Dick GJ, Tebo BM (2010) Microbial diversity and biogeochemistry of the Guaymas Basin deep-sea hydrothermal plume. *Environmental Microbiology*, **12**, 1334–1347.

Dixon P (2003) VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*, **3**, 927–930.

Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, Edrington TS (2008) Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiology*, **8**, 125.

Duan A, Wu G, Zhang Q, Liu Y (2006) New proofs of the recent climate warming over the Tibetan Plateau as a result of the increasing greenhouse gases emissions. *Chinese Science Bulletin*, **51**, 1396–1400.

Duffy JE, Macdonald KS, Rhode JM, Parker JD (2001) Grazer diversity, functional redundancy, and productivity in seagrass beds: an experimental test. *Ecology*, **82**, 2417–2434.

Ebersberger D, Werrnbter N, Niklaus PA, Kandeler E (2004) Effects of long term CO_2 enrichment on microbial community structure in calcareous grassland. *Plant and Soil*, **264**, 313–323.

Falkowski PG, Fenchel T, Delong EF (2008) The microbial engines that drive Earth's biogeochemical cycles. *Science*, **320**, 1034–1039.

FAO (2008) Are grasslands under threat? Brief analysis of FAO statistical data on pasture and fodder crops. Available at: http://www.fao.org/uploads/media/grass_-stats_1.pdf.

Giller PS, Hillebrand H, Berninger UG *et al.* (2004) Biodiversity effects on ecosystem functioning: emerging issues and their experimental test in aquatic environments. *Oikos*, **104**, 423–436.

de Goede RGM, Verschoor BC, Pronk TE, Brussaard L (2002) Could plant-feeding nematodes affect the competition between grass species during succession in grasslands under restoration management? *Journal of Ecology*, **90**, 753–761.

Grime JP (1997) Ecology – biodiversity and ecosystem function: the debate deepens. *Science*, **277**, 1260–1261.

Groffman P, Rice C, Tiedje J (1993) Denitrification in a tallgrass prairie landscape. *Ecology*, **74**, 855–862.

Guitian R, Bardgett RD (2000) Plant and soil microbial responses to defoliation in temperate semi-natural grassland. *Plant and Soil*, **220**, 271–277.

Harrison KA, Bardgett RD (2004) Browsing by red deer negatively impacts on soil nitrogen availability in regenerating native forest. *Soil Biology & Biochemistry*, **36**, 115–126.

Hazen TC, Dubinsky EA, DeSantis TZ *et al.* (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science*, **330**, 204–208.

He ZL, Gentry TJ, Schadt CW *et al.* (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME Journal*, **1**, 67–77.

He ZL, Xu MY, Deng Y *et al.* (2010a) Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO2. *Ecology Letters*, **13**, 564–575.

He ZL, Deng Y, Van Nostrand JD *et al.* (2010b) GeoChip 3.0 as a high-throughput tool for analyzing microbial community composition, structure and functional activity. *ISME Journal*, **4**, 1167–1179.

Head IM, Hiorns WD, Embley TM, McCarthy AJ, Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *Journal of General Microbiology*, **139** (Pt 6), 1147–1153.

Hess M, Sczyrba A, Egan R *et al.* (2011) Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science*, **331**, 463–467.

Hoffmann C, Funk R, Wieland R, Li Y, Sommer M (2008) Effects of grazing and topography on dust flux and deposition in the Xilingele grassland, Inner Mongolia. *Journal of Arid Environments*, **72**, 792–807.

Holland EA, Detling JK (1990) Plant-response to herbivory and belowground nitrogen cycling. *Ecology*, **71**, 1040–1049.

Holt JA (1997) Grazing pressure and soil carbon, microbial biomass and enzyme activities in semi-arid northeastern Australia. *Applied Soil Ecology*, **5**, 143–149.

Hooper DU, Vitousek PM (1997) The effects of plant composition and diversity on ecosystem processes. *Science*, **277**, 1302–1305.

Huber JA, Welch DBM, Morrison HG, Huse SM, Neal PR, Butterfield DA, Sogin ML (2007) Microbial population structures in the deep marine biosphere. *Science*, **318**, 97–100.

Hütsch BW, Webster CP, Powlson DS (1994) Methane oxidation in soil as affected by land use, soil pH and N fertilization. *Soil Biology & Biochemistry*, **26**, 1613–1622.

Jesus ED, Marsh TL, Tiedje JM, Moreira FMD (2009) Changes in land use alter the structure of bacterial communities in Western Amazon soils. *ISME Journal*, **3**, 1004–1011.

Kemper N (2008) Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicator*, **8**, 1–13.

Klein JA, Harte J, Zhao XQ (2004) Experimental warming causes large and rapid species loss, dampened by simulated grazing, on the Tibetan Plateau. *Ecology Letters*, **7**, 1170–1179.

Klein JA, Harte J, Zhao XQ (2007) Experimental warming, not grazing, decreases rangeland quality on the Tibetan Plateau. *Ecological Applications*, **17**, 541–557.

Kohler F, Hamelin J, Gillet F, Gobat JM, Buttler A (2005) Soil microbial community changes in wooded mountain pastures due to simulated effects of cattle grazing. *Plant and Soil*, **278**, 327–340.

Kolbl A, Steffens M, Wiesmeier M *et al.* (2011) Grazing changes topography-controlled topsoil properties and their interaction on different spatial scales in a semi-arid grassland of Inner Mongolia, P.R. China. *Plant and Soil*, **340**, 35–58.

Le Roux X, Poly F, Currey P *et al.* (2008) Effects of aboveground grazing on coupling among nitrifier activity, abundance and community structure. *ISME Journal*, **2**, 221–232.

Liang Y, Van Nostrand JD, Deng Y *et al.* (2011) Functional gene diversity of soil microbial communities from five oil-contaminated fields in China. *ISME Journal*, **5**, 403–413.

Liu W, Zhang XZ, Zhang Z, Zhang YH (2010) Engineering of Clostridium phytofermentans Endoglucanase Cel5A for improved thermostability. *Applied and Environment Microbiology*, **76**, 4914–4917.

Lu Z, Deng Y, Van Nostrand JD *et al.* (2012) Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. *ISME Journal*, **6**, 451–460.

Luton PE, Wayne JM, Sharp RJ, Riley PW (2002) The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology*, **148**, 3521–3530.

McAllister TA, Topp E (2012) Role of livestock in microbiological contamination of water: commonly the blame, but not always the source. *Animal Frontiers*, **2**, 17–27.

Mermelstein LD, Welker NE, Bennett GN, Papoutsakis ET (1992) Expression of cloned homologous fermentative genes in Clostridium acetobutylicum ATCC 824. *Biotechnology*, **10**, 190–195.

Mills A, Adl MS (2006) The effects of land use intensification on soil biodiversity in the pasture. *Canadian Journal of Plant Science*, **86**, 1339–1343.

Monaghan R, Barraclough D (1993) Nitrous oxide and dinitrogen emissions from urine-affected soil under controlled conditions. *Plant and Soil*, **151**, 127–138.

Morin PJ, McGrady-Steed J (2004) Biodiversity and ecosystem functioning in aquatic microbial systems: a new analysis of temporal variation and species richness-predictability relations. *Oikos*, **104**, 458–466.

Pointing SB, Chan Y, Lacap DC, Lau MC, Jurgens JA, Farrell RL (2009) Highly specialized microbial diversity in hyper-arid polar desert. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 19964–19969.

Qiu J (2008) China: the third pole. *Nature*, **454**, 393–396.

Radl V, Gattinger A, Chronakova A *et al.* (2007) Effects of cattle husbandry on abundance and activity of methanogenic archaea in upland soils. *ISME Journal*, **1**, 443–452.

Roux XL, Bardy M, Loiseau P, Louault F (2003) Stimulation of soil nitrification and denitrification by grazing in grasslands: do changes in plant species composition matter? *Oecologia*, **137**, 417–425.

Ruess RW, Mcnaughton SJ (1987) Grazing and the dynamics of nutrient and energy regulated microbial processes in the Serengeti grasslands. *Oikos*, **49**, 101–110.

Sala OE, Chapin FS 3rd, Armesto JJ *et al.* (2000) Global biodiversity scenarios for the year 2100. *Science*, **287**, 1770–1774.

Seagle SW, McNaughton S (1993) Simulated effects of precipitation and nitrogen on Serengeti grassland productivity. *Biogeochemistry*, **22**, 157–178.

Sebaihia M, Peck MW, Minton NP *et al.* (2007) Genome sequence of a proteolytic (Group I) Clostridium botulinum strain Hall A and comparative analysis of the clostridial genomes. *Genome Research*, **17**, 1082–1092.

Simek M, Hynst J, Brucek P, Petersen SO (2007) High fluxes but different patterns of nitrous oxide and carbon dioxide emissions from soil in a cattle overwintering area. *Agriculture Ecosystems & Environment*, **120**, 269–279.

Simek M, Brucek P, Hynst J (2009) Long-term animal impact modifies potential production of N(2)O from pasture soil. *Biology and Fertility of Soils*, **46**, 27–36.

Thompson K, Askew AP, Grime JP, Dunnett NP, Willis AJ (2005) Biodiversity, ecosystem function and plant traits in mature and immature plant communities. *Functional Ecology*, **19**, 355–358.

Torsvik V, Ovreas L, Thingstad TF (2002) Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Science*, **296**, 1064–1066.

Tracy BF, Frank DA (1998) Herbivore influence on soil microbial biomass and nitrogen mineralization in a northern grassland ecosystem: yellowstone National Park. *Oecologia*, **114**, 556–562.

Van Nostrand JD, Wu WM, Wu LY *et al.* (2009) GeoChip-based analysis of functional microbial communities during the reoxidation of a bioreduced uranium-contaminated aquifer. *Environmental Microbiology*, **11**, 2611–2626.

Venter JC, Remington K, Heidelberg JF *et al.* (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science*, **304**, 66–74.

Wang KH, McSorley R, Bohlen P, Gathumbi SM (2006a) Cattle grazing increases microbial biomass and alters soil nematode communities in subtropical pastures. *Soil Biology & Biochemistry*, **38**, 1956–1965.

Wang SP, Ma XZ, Wang YF, Jiang GM, Nyren P (2006b) Short-term effects of sheep excrement on carbon dioxide, nitrous oxide and methane fluxes in typical grassland of Inner Mongolia. *New Zealand Journal of Agricultural Research*, **49**, 285–297.

Wang SP, Lin XW, Ma XZ *et al.* (2009) Fluxes of CO(2), CH(4), and N(2)O in an alpine meadow affected by yak excreta on the Qinghai-Tibetan plateau during summer grazing periods. *Soil Biology & Biochemistry*, **41**, 718–725.

Wang SP, Luo CY, Xu GP *et al.* (2010) Effect of warming and grazing on litter mass loss and temperature sensitivity of litter and dung mass loss on the Tibetan plateau. *Global Change Biology*, **16**, 1606–1617.

Wardle DA, Bonner KI, Barker GM *et al.* (1999) Plant removals in perennial grassland: vegetation dynamics, decomposers, soil biodiversity, and ecosystem properties. *Ecological Monographs*, **69**, 535–568.

Wolf B, Zheng XH, Brueggemann N *et al.* (2010) Grazing-induced reduction of natural nitrous oxide release from continental steppe. *Nature*, **464**, 881–884.

Wright AD, Auckland CH, Lynn DH (2007) Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. *Applied and Environment Microbiology*, **73**, 4206–4210.

Yamulki S, Jarvis SC, Owen P (1998) Nitrous oxide emissions from excreta applied in a simulated grazing pattern. *Soil Biology & Biochemistry*, **30**, 491–500.

Yang H, Lu Q, Wu B, Zhang J, Lin Y (2006) Vegetation diversity and its application in sandy desert revegetation on Tibetan Plateau. *Journal of arid environments*, **65**, 619–631.

Yao J, Yang B, Yan P (2006) Analysis on habitat variance and behaviour of Bos gruiens in China. *Acta Prataculturae Sinica*, **15**, 124–128.

Yeates GW, Bardgett RD, Cook R, Hobbs PJ, Bowling PJ, Potter JF (1997) Faunal and microbial diversity in three Welsh grassland soils under conventional and organic management regimes. *Journal of Applied Ecology*, **34**, 453–470.

Zhao L, Li Y, Xu S, Zhou H, Gu S, Yu G, Zhao XQ (2006) Diurnal, seasonal and annual variation in net ecosystem CO2 exchange of an alpine shrubland on Qinghai-Tibetan plateau. *Global Change Biology*, **12**, 1940–1953.

Zheng D, Zhang Q, Wu S (2000) *Mountain Geoecology and Sustainable Development of the Tibetan Plateau*. Kluwer Academic, Norwell, MA.

Zhou J, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. *Applied and Environment Microbiology*, **62**, 316–322.

Zhou J, Wu L, Deng Y *et al.* (2011) Reproducibility and quantitation of amplicon sequencing-based detection. *ISME Journal*, **5**, 1303–1313.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The grazing effect on overall microbial community structure and environmental variables as examined by the dissimilarity test of adonis.

Table S2. Summary of environmental variable measurements.

Figure S1. Hierarchical clustering analysis of microbial communities for all of the sites.

Figure S2. The normalized signal intensity of the significantly decreased mcrA genes.