

ORIGINAL ARTICLE

The microbe-mediated mechanisms affecting topsoil carbon stock in Tibetan grasslands

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Warming has been shown to cause soil carbon (C) loss in northern grasslands owing to accelerated microbial decomposition that offsets increased grass productivity. Yet, a multi-decadal survey indicated that the surface soil C stock in Tibetan alpine grasslands remained relatively stable. To investigate this inconsistency, we analyzed the feedback responses of soil microbial communities to simulated warming by soil transplant in Tibetan grasslands. Whereas microbial functional diversity decreased in response to warming, microbial community structure did not correlate with changes in temperature. The relative abundance of catabolic genes associated with nitrogen (N) and C cycling decreased with warming, most notably in genes encoding enzymes associated with more recalcitrant C substrates. By contrast, genes associated with C fixation increased in relative abundance. The relative abundance of genes associated with urease, glutamate dehydrogenase and ammonia monooxygenase (*ureC*, *gdh* and *amoA*) were significantly correlated with N₂O efflux. These results suggest that unlike arid/semiarid grasslands, Tibetan grasslands maintain negative feedback mechanisms that preserve terrestrial C and N pools. To examine whether these trends were applicable to the whole plateau, we included these measurements in a model and verified that topsoil C stocks remained relatively stable. Thus, by establishing linkages between microbial metabolic potential and soil biogeochemical processes, we conclude that long-term C loss in Tibetan grasslands is ameliorated by a reduction in microbial decomposition of recalcitrant C substrates.

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Introduction

Global warming has a wide influence on terrestrial ecosystems, particularly in polar or alpine ecosystems, where it is predicted to be more pronounced

(Walther *et al.*, 2002). A number of field and modeling studies have indicated that climate warming causes soil carbon (C) loss in northern ecosystems (Melillo *et al.*, 2002; Feng *et al.*, 2008; Ise *et al.*, 2008); yet, a plateau-wide survey of topsoil C stocks in Tibetan grasslands from 1980 to 2004 suggested that it was remarkably stable (Yang *et al.*, 2009) in spite of climatic warming in this region, which is currently three times the global average (Li and Tang, 1988). This inconsistency suggests that biogeochemical processes must be stabilizing these C reservoirs; here we aim to elucidate the genetic

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mechanisms underpinning such activity. As microbes have an important role in mediating biogeochemical processes (Falkowski *et al.*, 2008), we hypothesize that differential microbial functional capacity in C degradation and fixation might be responsible for this inconsistency.

The effects of climate warming can be simulated using artificial warming (Zhang *et al.*, 2005), observational investigations along a temperature gradient (Dunne *et al.*, 2004; Yang *et al.*, 2014), and/or common garden transplant experiments (Waldrop and Firestone, 2006; Lazzaro *et al.*, 2011; Zhao *et al.*, 2014). Although artificial warming is straightforward and widely adopted, it can decrease soil moisture content, which conflicts with how we expect alpine and polar regions to respond to climate warming, as they will likely experience higher soil moisture owing to glacial and permafrost melt. Recently, soil transplants have been used to simulate the impact of climate change on terrestrial ecosystems (Balsler and Firestone, 2005; Breeuwer *et al.*, 2010; De Frenne *et al.*, 2011; Lazzaro *et al.*, 2011; Vanhala *et al.*, 2011), with significant changes in microbial community structure observed with increased temperature (Vanhala *et al.*, 2011). However, there are only limited studies, which provide evidence of the responses of microbial functional potential to soil warming.

Here we used common garden soil transplantation in alpine grassland on the Tibetan plateau, along an elevation gradient of 3200, 3400, 3600 and 3800 m above the sea level. Soil was transplanted from higher elevation to lower elevation plots to simulate warming, and microbial community structure and functions were profiled to determine what were the potential mechanisms underpinning C and nitrogen (N) metabolism, how microbially mediated environmental processes (for example, gas flux) correlated with changes in microbial gene abundance, and whether these changes could be modeled to extrapolate observed processes.

Materials and methods

The study site and experiment design

Four different elevations (3200, 3400, 3600 and 3800 m) along the southern slope of the Qilian Mountains, Qinghai, China, located in Haibei Alpine Meadow Ecosystem Research Station (37°37'N, 101°12'E) of the Northeastern Tibet Plateau, were used for the transplant experiment. The region is dominated by discontinuous permafrost (Zhuang *et al.*, 2010), and the mean annual air temperature and rainfall precipitation is -1.7°C and 560 mm, respectively (Zhao *et al.*, 2006). The main soil type is Mat Cryic Cambisols, and the dominant aboveground vegetation is C_3 herbal species. Soil pH at the depths of 10 and 20 cm is 7.3 and 7.4, respectively.

Experimental plots with sizes of 1 m length \times 1 m width \times 0.3 m depth were set up in May 2007. Along an elevation gradient of 3200, 3400, 3600 and

3800 m above the sea level, soil was transplanted in triplicates downward from higher to lower sites, resulting in a total of 18 transplanted plots (namely, 3800T3600, 3800T3400, 3800T3200, 3600T3400, 3600T3200 and 3400T3200). To minimize the disturbance to aboveground vegetation, four intact soils with the size of 0.5 m length \times 0.5 m width \times 0.3 m depth were dug out of the ground as a whole and pieced together to form a plot. Three plots at the elevations of 3400, 3600 and 3800 m were mock transplanted as they were dug out and then put back to the same places to serve as the control.

Soil samples were collected in August 2009 and used for GeoChip 4.0 experiments and environmental variable measurements. Three soil cores with a diameter of 1.5 cm at the depth of 0–20 cm were taken randomly at each plot. Then soil samples were transported to laboratory with ice and sieved with 2 mm mesh to remove visible grassroots and stones. Soil samples for GeoChip experiments were kept at -80°C until DNA extraction, and soil samples for environmental variable measurements were kept at 4°C or -20°C .

Environmental variable measurements

Soil temperature was measured at the depths of 5, 10 and 20 cm using type-K thermocouples (Campbell Scientific, Logan, UT, USA) coupled to a CR1000 datalogger, whereas soil moisture was recorded every 30 min at the depths of 5 and 10 cm with time domain reflectometry (Model Diviner-2000, Sentek Pty Ltd, Stepney, SA, Australia). Soil biogeochemical variables were measured as previously described (Yang *et al.*, 2014). In brief, total organic C (TOC) and total N (TN) were measured by a TOC-5000 A analyzer (Shimadzu Corp., Kyoto, Japan) and a Vario EL III Elemental Analyzer (Elementar, Hanau, Germany), respectively, as described previously (Ryba and Burgess, 2002; Stevens *et al.*, 2012). $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ and greenhouse gas (CO_2 , N_2O and CH_4) effluxes were analyzed with a FIAstar 5000 Analyzer (FOSS, Hillerd, Denmark) and a gas chromatography (HP Series 4890D, Hewlett Packard, Wilmington, DE, USA), respectively. C/N ratio was calculated, dividing TOC by total N.

Soil bulk density at the depth of 0–20 cm was estimated by an empirical equation (1) using the corresponding TOC data at depths of 0–10 cm and 10–20 cm, whereas soil organic C density at the depth of 0–20 cm was calculated using equation (2), in which n was 2 for the two layers with soil thickness of 10 cm (Yang *et al.*, 2009).

$$BD = 0.3 + 1.28 \exp(-0.01724 \text{TOC}) \quad (1)$$

$$\text{SOCD} = \sum_{i=1}^n L_i \times BD_i \times \text{TOC}_i \times (1 - M_i) / 100 \quad (2)$$

where SOCD, L , BD , TOC and M were soil organic C density (kg Cm^{-2}), soil thickness (cm), bulk density (g cm^{-3}), TOC (g kg^{-1}) and percentage of the > 2 mm

fraction, respectively. In our C model, changes of SOCD were calculated by the differences of TOC between control and transplanted samples. The SOCD distribution was displayed using histogram, and the average SOCD change and its uncertainty were estimated by commanding 'one.boot' in the R package simpleboot (Peng, 2008).

Vegetation variables were measured in a selected quadrat in the plot. Vegetation species, density, biomass and average height were recorded according to an established protocol (Klein *et al.*, 2007). Plant aboveground biomass was estimated using a non-destructive sampling method (Klein *et al.*, 2007).

DNA extraction and GeoChip analysis

DNA extraction, purification, labeling, hybridization to GeoChip 4.0, raw data processing and statistical analyses were described in recent studies (Yang *et al.*, 2013; Chu *et al.*, 2014; Gao *et al.*, 2014). In brief, DNA was extracted from soil samples at the depth of 0–20 cm using FastDNA spin kit for soil (MP Biomedical, Carlsbad, CA, USA) and then precipitated and dissolved in nuclease-free water. After labeling with fluorescent dye Cy-5, 1.0 µg DNA was hybridized with GeoChip 4.0 in a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA) and scanned by a NimbleGen MS200 scanner (Roche, Madison, WI, USA). GeoChip data were normalized by relative abundance, that is, each spot divided by total intensity of its microarray and then multiplied with average value of microarray's total intensity. After that, the data were transformed by natural logarithm.

Results

The transplant effects on vegetation, soil variables and microbial community

Nearly all soil transplants showed a significant increase ($P=0.006$) in total aboveground vegetation biomass, total vegetation coverage and vegetation species number (Supplementary Table S1). Similarly, a significant increase in the concentration of TOC, total N and ammonia at the soil depths of 0–10 and 10–20 cm was also observed (Supplementary Table S1). A significant increase was also observed in the efflux of CO₂ and N₂O, whereas CH₄ efflux remained unchanged. A significant positive correlation between CO₂ efflux and TN_{10 cm} ($r=0.69$, $P<0.001$) was observed, as recorded previously (Bragazza *et al.*, 2006).

Overall microbial functional diversity (Shannon and Simpson indices) was significantly lower in transplanted samples than control samples ($P<0.003$; Supplementary Table S2). Also, microbial community structures were clearly changed, with transplanted samples showing significantly different community structure compared with their in-place controls (Supplementary Figure. S1; *Adonis* $P<0.081$). In addition, changes in microbial

community structure did not show linear correlations with geographic distance or changes in temperature (Supplementary Figure. S2). For example, both distance and change in temperature were less between the 3800 and 3600 m sites when compared with the 3800 and 3400 m sites, yet microbial community structure in the soil transplanted from 3800 to 3600 m showed a significantly greater change compared with soil transplanted from 3800 to 3400 m. The functional gene structure of the microbial communities was significantly ($P=0.001$) correlated with environmental variables, but not with geographic distance or temperature (Table 1). Interestingly, a similar non-linear response has been observed in previous soil warming experiments (Xiong *et al.*, 2014).

The transplant effects on N cycle genes

Overall, genes associated with N cycle demonstrated significantly lower relative abundance ($P<0.050$) in transplanted samples. However, specific genes showed differing responses, for example, the relative abundance of *ureC* increased, whereas *gdh* decreased in transplanted samples (Figure 1a). The combined effect of these two changes potentially could result in an increase in urea ammonification and hence the N mineralization, because *ureC* protein converts urea into ammonia, and the *gdh* protein converts α -ketoglutarate and ammonia to glutamate. Consistently, the ammonia content and pH were higher at warmer sites (Supplementary Table S1), thus microbe-available ammonia was increased, as microbes preferentially used NH₃-N but not NH₄⁺-N (He *et al.*, 2012). Almost all of other N cycle genes were less abundant at warming sites except the nitrate reduction gene *narG*. In accordance, the NO₃⁻-N content was lower and TN content was higher at the warming sites (Supplementary Table S1). Notably, the lower abundance of N fixation gene *nifH* at warming sites was consistent with a previous finding, showing that increased N suppressed N fixers (Walker *et al.*, 2008).

Pearson correlation analyses showed that N₂O efflux had positive correlations with *ureC* ($P=0.001$) and ammonium-oxidizing gene *amoA* ($P=0.029$) but a negative correlation with *gdh*

Table 1 Effects of environmental variables, temperature and geographic distance on microbial community by partial Mantel test

	Environmental variable ^a	Temperature	Geographic distance
Statistic <i>r</i>	0.417	-0.294	-0.310
Significance <i>P</i>	0.001***	1.000	1.000

^aEnvironmental variables include soil geochemistry and vegetation variables. Temperature variables include air and soil temperature. Geographic distances are calculated based on longitude, latitude and elevation of each site. *** $P<0.001$.

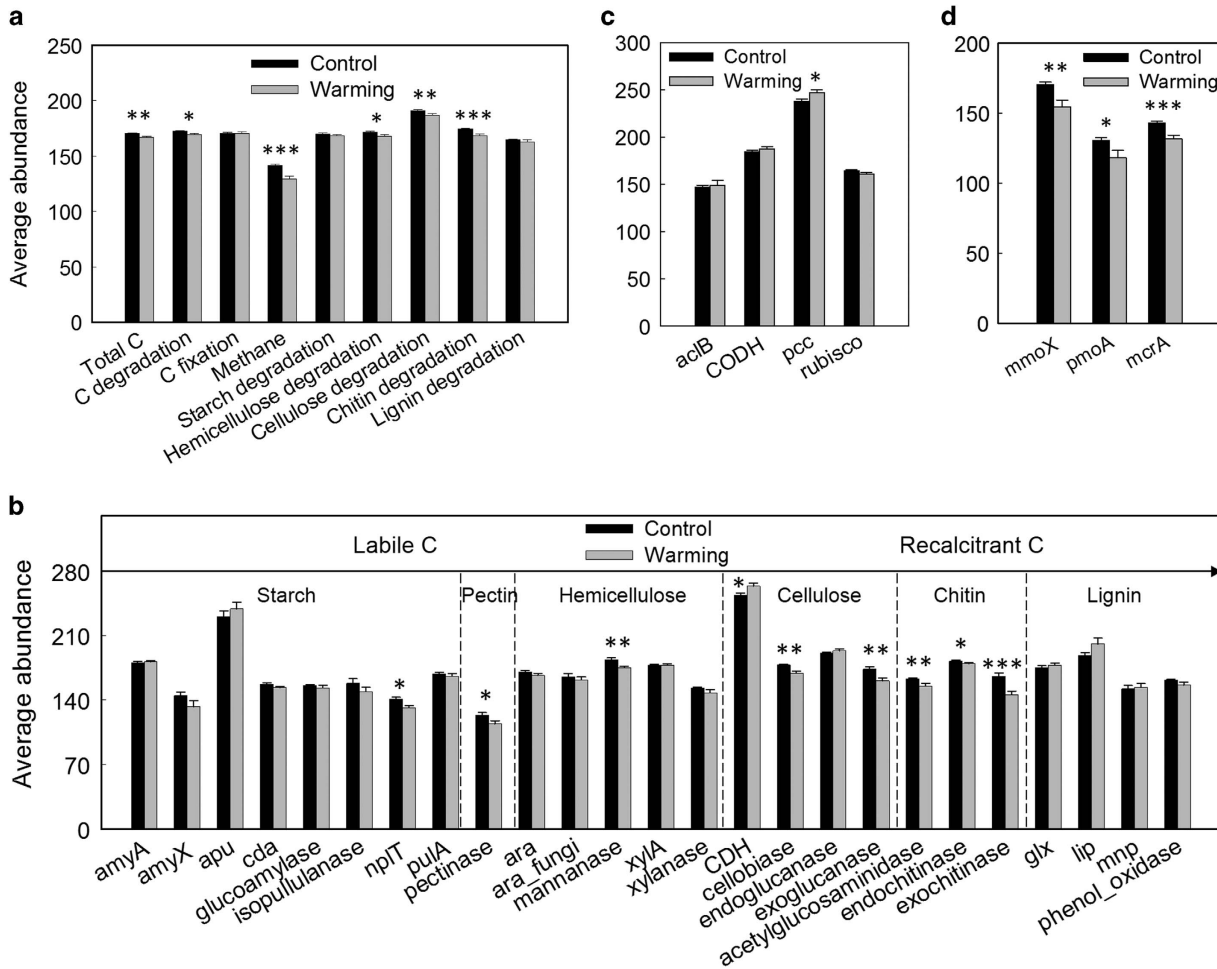


Figure 2 The warming effect on C cycle genes. Average abundances of (a) overall C functional group, (b) C degradation, (c) C fixation and (d) CH₄ cycle genes are compared between warming and control sites. Error bars represent standard error ($n = 18$). The differences between warming and control sites were analyzed by two-tailed paired t -tests. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

would have resulted in no change in net CH₄ efflux (Supplementary Table S1). Almost all of *mcrA* genes were derived from uncultured *Euryarchaeota* or Archaea, whereas almost all of *mmoX* and *pmoA* genes were derived from uncultured bacteria.

The transplant effects on selected stress genes

Cold shock genes may be important for coping with the periodically cold alpine environment. Under the warming condition, the total abundance of cold shock genes (*cspA*, *cspB*, *desR* and *desK*), derived from a wide range of phyla including *Actinobacteria*, *Firmicutes* and *Proteobacteria*, significantly decreased (Supplementary Figure. S3), suggesting that cold stress in the alpine grassland was ameliorated by warming. In addition, stress genes responsive to P limitation (*phoAB* and *pstABC*) and N limitation (*glnR*) were also decreased. Accordingly, TN content was significantly greater at the warming sites (Supplementary Table S1).

Environmental variables linking to microbial communities

To identify environmental variables linking to microbial community structure, automatic forward selection and variance inflation factors were used to select variables independent of other variables, resulting in 12 variables (annual air temperature, soil temperature, soil moisture, water content, soil pH, NO₃⁻-N, ammonia, TOC, TN, vegetation biomass, total coverage of plants and species number). Subsequently, canonical correspondence analysis was performed, resulting in a significant ($P = 0.005$) model (Figure 3). A total of 38.6% variance by the first two axes was constrained in the canonical correspondence analysis, and the results were supported by Mantel test at $P < 0.050$ (Table 2). Moisture, ammonia, TOC_{10 cm}, TOC_{20 cm}, TN_{20 cm}, C/N ratio, vegetation biomass and N₂O appeared to be strongly linked to microbial community structure. Collectively, these results suggested that microbial community was correlated

with soil C and N storage, which would support a specific influence.

Modeling of topsoil C stock

To examine whether our observations were representative of Tibetan grasslands at the plateau level, we fed SOC measurements at the study site to a satellite-based SOC model (Yang *et al.*, 2009) and examined whether it was consistent with the findings in the plateau-wide survey of topsoil C stock. On average, SOC stock in the top 20 cm depth of the four elevations in the alpine grasslands was 5.920 kg Cm^{-2} . SOC changes for the downward transplanting experiment were estimated at 0.072 kg Cm^{-2} , ranging from $-0.100 \text{ kg Cm}^{-2}$ to 0.300 kg Cm^{-2} . Uncertainty analysis revealed SOC changes fluctuated between $-0.003 \text{ kg Cm}^{-2}$ and 0.104 kg Cm^{-2} at the 99% confidence level using basic bootstrap method (Figure 4), which validated the results of the survey that topsoil C stocks remained relatively stable but slightly increased at the plateau level.

Discussion

The effects of warming on terrestrial C and N pools of alpine grasslands remain elusive. Also, it has been documented that temperature affects microbial community structure and/or biomass in the alpine meadow (Lipson *et al.*, 2002). Therefore, soil transplant was conducted in this study to examine topsoil C and N stocks in alpine grasslands and their linkages with microbial community. Four-years of warming in alpine grassland, as simulated by soil transplant, changed the relative abundances of microbial N and C cycle genes, which were concomitant with soil N and C content, and the

identified mechanisms provided explanation for the stable but slightly increased trends in topsoil C stock at the plateau level. Temperature-dependent increases in vegetation biomass associated with tundra, grassland and forest ecosystems (Dukes *et al.*, 2005) were also verified by the current study. In addition, we demonstrated that the soil microbial community structure was changed as a result of warming, which was consistent with previous studies (Xiong *et al.*, 2014). However, while the majority of these studies have explored the impact of temperature on phylogenetic and taxonomic structure of microbial communities, this study has explicitly determined the influence of transplant-associated warming on the microbial functional potential, and linked this directly to the changes in C and N pools in these soils.

The total aboveground vegetation biomass, total vegetation coverage and species number increased in transplant soils (Supplementary Table S1), which had also been well documented elsewhere that warming stimulated vegetation growth in cold environments (Natali *et al.*, 2012; Baldwin *et al.*, 2014; DeMarco *et al.*, 2014). As Mantel test showed that only total aboveground vegetation biomass was correlated with microbial community (Table 2), increased vegetation biomass could lead to an increase of fresh soil C input from litter, which might shift microbial community away from utilizing recalcitrant C. In addition, soil C loss through CO_2 efflux was observed, as shown previously (Lin *et al.*, 2009; Luo *et al.*, 2009; Zhou *et al.*, 2012). However, no significant correlation between CO_2 efflux and C cycle genes was observed here, which could be interpreted as suggesting that microbial heterotrophic respiration was not the major contributor of soil CO_2 efflux. Rather, it is likely that the increase of soil CO_2 efflux was caused by soil autotrophic respiration, yet, we have no direct evidence to support this hypothesis. This is in direct contrast to arid/semiarid grasslands, where >50% of soil respiration was performed by heterotrophic activity (Zhou *et al.*, 2012). The alpine grassland is typically a cold and wet climate, which substantially differs from the hot and dry climate of arid/semiarid grassland, and as both temperature and moisture can directly influence heterotrophic respiration (Xu *et al.*, 2004), it is likely to be significantly lower in alpine soils, leading to greater net C storage.

Total C, N and ammonia contents in arid/semiarid grasslands were reduced under warming owing to microbial activity (Zhou *et al.*, 2012), yet in the present study, ammonia and TN contents were significantly higher under warming. The differential increase of the abundance of *ureC* and the decrease of *gdh* may shuttle urea metabolism toward ammonia biosynthesis, resulting in an increased concentration of soil ammonia. However, we cannot assess the changes of genes associated with proteinases and deaminases, also important for N mineralization

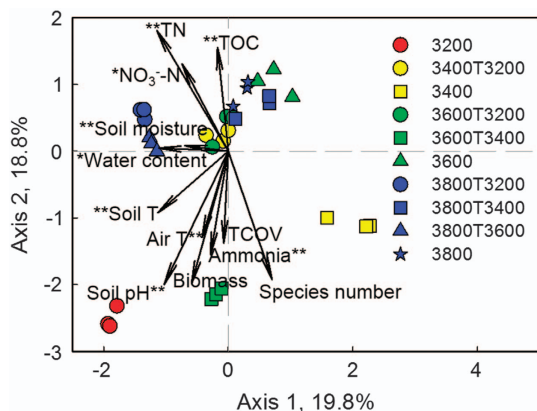


Figure 3 Linkages between microbial community and environmental variables. Canonical correspondence analysis (CCA) displayed microbial community (symbols) and environmental variables (arrows). The value of the axis is the variance percentage explained for the axis. The significance of each environmental variable was calculated by ANOVA. ** $P < 0.01$, * $P < 0.05$. T, temperature; TOC, total soil organic C; TN, total soil N; TCOV, total coverage of vegetation.

Table 2 Linkages between microbial community and environmental variables by Mantel tests

Environmental variable (unit)	r-value	Environmental variable (unit)	r-value
T_5 ^a (°C)	-0.05	TN_20 (g kg ⁻¹)	0.168*
T_10 (°C)	-0.007	C/N_10	0.439**
Annual air T (°C)	-0.093	C/N_20	0.218**
Soil pH	0.118	SIN (mg kg ⁻¹)	0.136
Soil moisture (%)	0.171* ^b	Vegetation biomass (g)	0.264*
NO ₃ -N (mg kg ⁻¹)	-0.032	Total coverage of vegetation (%)	-0.092
Ammonia (mg kg ⁻¹)	0.235*	Vegetation species number	0.094
TOC_10 (g kg ⁻¹)	0.190**	CH ₄ flux (μg m ⁻² h ⁻¹)	0.071
TN_10 (g kg ⁻¹)	0.003	CO ₂ flux (mg m ⁻² h ⁻¹)	0.003
TOC_20 (g kg ⁻¹)	0.408**	N ₂ O flux (μg m ⁻² h ⁻¹)	0.342***

^aAbbreviations: SIN, Soil inorganic N; T, temperature; TOC, Total soil organic C; TN, Total soil N; 5, 10 or 20, soil variables measured at the depths of 5, 10 or 20 cm.

^bSignificance: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

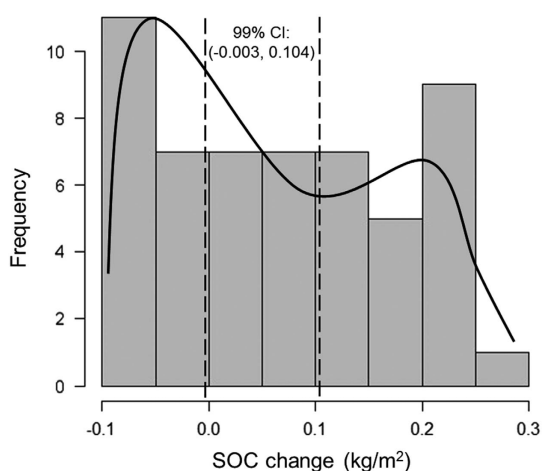


Figure 4 Topsoil C stock modeling of the Tibetan grasslands based on a satellite-based approach (Yang *et al.*, 2009). Bar and solid line represents the frequency and distribution trend of SOC change in Tibetan grasslands, respectively. The interval between dash lines represents the 99% confidence interval (CI) calculated based on basic bootstrap method.

(Geisseler *et al.*, 2010), as GeoChip 4.0 does not contain their probes. All of the three genes associated with chitin degradation, endochitinase, exochitinase and acetylglucosaminidase, decreased in abundance (Figure 2b), suggestive of a decreased need for microbes to mine chitin for its N demand. The increase of TN content was significantly correlated to the changing microbial community structure. Together, these results indicate that warming effects on the N cycle substantially differ between alpine and arid/semiarid grasslands. Notably, a recent work at our study site showed that biological N₂-fixation by leguminous plants made a very small contribution to N cycle as its air temperatures during the growth season fell well below the optimum range of 20–35 °C for nitrogenase activity (Yang *et al.*, 2011).

Nitrification appeared to contribute significantly to N₂O efflux in the Tibetan grassland, as total abundances of *amoA* and the preceding step (*ureC* and *gdh*) were correlated to N₂O efflux, which established a linkage between microbial metabolic

potential and soil biogeochemical processes. This is supported by previous studies, suggesting that the nitrification process could be a dominant mechanism of grassland N₂O emission (Klemetsson *et al.*, 1988; Yang *et al.*, 2014). Although messenger RNA analysis is needed to determine whether these genes were being actively transcribed, recent studies have demonstrated that analyzing the gene abundance using DNA can provide strong correlations between N cycling genes and greenhouse gas (N₂O) emissions (Morales *et al.*, 2010; Liu *et al.*, 2014; Zhao *et al.*, 2014).

Using soil C model in this study, topsoil C stocks were predicted to remain relatively stable under climate warming, validating a soil C survey of the Tibetan grasslands (Yang *et al.*, 2009). A general increase of vegetation biomass by climate warming has been predicted and observed worldwide (Elmendorf *et al.*, 2012), and this ubiquity means that inconsistencies in soil C stock across different ecosystems may not be wholly driven by plant-derived C inputs. The microbial community must have an important role in controlling topsoil C stocks. Enhanced C sequestration through reducing microbial functional potentials in degrading C substrates, particularly recalcitrant C substrates, appeared to be involved in stabilizing topsoil C stock content in Tibetan grasslands.

Recently, initial efforts to include microbial processes in climate modeling have resulted in improved soil C projections at the global scale (Wieder *et al.*, 2013). However, the predictions made for the Tibetan plateau substantially deviated from actual observations, emphasizing the need to develop a high-resolution model that explicitly includes microbial community structure and functional dynamics. The inclusion of microbial gene abundances for key metabolic pathways into climate models will likely significantly improve regional accuracy in C dynamic predictions. The mechanisms of C and N regulation identified here provide valuable insights for these predictions.

In conclusion, our findings unveil negative feedbacks that help maintain terrestrial C and N storages,

which substantially differ from what is known for arid/semiarid grasslands. The incorporation of microbial functional genes, which has not been explicitly considered in C-climate models, will be useful to further improve the predictions of ecosystem feedbacks to climate warming.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

This study was conceived and led by SW, JZ and YY, HY, QL, XL and YY carried out GeoChip experiments and environmental measurements, YH, MW, XS and LW performed the analytical work, HY, SW, JG, JZ and YY wrote the manuscript. All authors discussed the results and their implications and commented on the manuscript as it progressed.

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