Yu, J., Bing, H., Chang, R., Cui, Y., **Shen, G.**, Wang, X., Zhang, S., Fang, L. (2022):
Microbial metabolic limitation response to experimental warming along an altitudinal gradient in alpine grasslands, eastern Tibetan Plateau
Catena **214** , art. 106243

http://dx.doi.org/10.1016/j.catena.2022.106243

Microbial metabolic limitation response to experimental warming along an altitudinal gradient in alpine grasslands, eastern Tibetan Plateau

Jialuo Yu [a, b], Haijian Bing [c], Ruiying Chang [c], Yongxing Cui [d], Guoting Shen [e], Xiangxiang Wang [a], Shangpeng Zhang [a, b], Linchuan Fang [a, f, *]

[a] State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation CAS and MWR, Yangling 712100, PR China

[b] University of Chinese Academy of Sciences, Beijing 100049, PR China

[c] Key Laboratory of Mountain Surface Processes and Ecological Regulation, Institute of Mountain Hazards and Environment, Chinese Academy of Sciences, Chengdu 610041, PR China

[d] Sino-French Institute for Earth System Science, College of Urban and Environmental Sciences, Peking University, Beijing, 100871, China

[e] Department of Soil Ecology, Helmholtz Centre for Environmental Research–UFZ, Halle, Saale, Germany

[f] CAS Center for Excellence in Quaternary Science and Global Change, Chinese Academy of Sciences, Xian 710061, PR China

* Corresponding author, E-mail address: flinc629@hotmail.com

Abstract

Alpine grassland is an important carbon (C) reservoir in terrestrial ecosystems. The response of microbial metabolism to global warming is essential to ecosystem C sequestration, yet the patterns and drivers of microbial metabolism activities in the soils of alpine grassland under warming remain poorly understood. Here, according to soil enzymatic stoichiometry, we observed that microbial metabolism was limited by C and phosphorus (P) in the alpine grassland ecosystem, eastern Tibetan Plateau, and microbial C limitation increased with elevation from 3800 to 4200 m despite the topsoil (0-5 cm) or subsoil (5-10 cm). Experimental warming significantly decreased microbial C limitation in the topsoil, but not the subsoil. The significantly negative correlation between microbial C and P limitation in both topsoil and subsoil suggests that the alleviation of C limitation after warming may aggravate the microbial P limitation. Furthermore, the content and ratio of nutrients and microbial biomass mainly affected microbial C and P limitation in the alpine grassland, respectively. Our results indicated that climate warming could modulate microbial metabolic limitation in alpine grassland soils and thus soil C sequestration by regulating nutrient availability and microbial biomass. This study provides an insight into the microbial regulation of nutrient cycles global warming, which is helpful for ecosystem C cycling in alpine grassland.

Key words: Warming; Microbial nutrient limitation; Ecoenzymatic stoichiometry; Altitudinal gradient; Alpine grassland

1. Introduction

Alpine ecosystems with the large amounts of carbon (C) reserves are especially vulnerable to climate warming and have a profound impact on the C cycle of terrestrial ecosystems (Chen et al., 2016; Lv et al., 2020). The environmental changes caused by global warming will directly or indirectly affect the growth and metabolic process of soil microorganisms, which eventually regulate terrestrial C feedback to climate warming (Lu et al., 2013; Zhou et al., 2013). Numerous studies have shown that increasing temperature may accelerate the microbial decomposition efficiency and amplify C release from soils to the atmosphere (Bardgett et al., 2008; Crowther et al., 2016). In contrast, the increased C input from vegetation and the change of soil moisture after warming would promote negative C-climate feedback by affecting microbial growth and metabolism (Lu et al., 2013). Therefore, the uncertainties regarding the responses of microbial nutrient acquisition and metabolic characteristics after climate warming remain to be addressed.

Microbial decomposers are the dominant drivers of C cycle and nutrient transformation through the production of soil extracellular enzymes (Burns et al., 2013; Manzoni, 2017; Sinsabaugh et al., 2009; Zuccarini et al., 2020). The effect of global warming on soil organic matter (SOM) decomposition depends on the response of soil extracellular enzymes activities (EEAs) to temperature changes (Zheng et al., 2020), as temperature can alter soil EEAs and accelerate nutrient mineralization processes (Guan et al., 2020; Zuccarini et al., 2020). Increased soil nutrient availability after warming decreases the production of extracellular enzymes for acquiring nutrient, thus enhancing biomass growth (Dove et al., 2021). The balance between nutrient demands of microbe and nutrient availability is regulated by metabolic activities, which can be well indicated by soil extracellular enzyme stoichiometry (Sinsabaugh and Shah, 2012; Tapia-Torres et al., 2015). Soil microbial activities at high altitudes and latitudes may be highly sensitive to global warming (Zhang et al., 2015). Several studies have indicated that EEAs in colder regions are more sensitive to temperature increase than those in warmer regions (Koch et al., 2007; Wallenstein et al., 2009). A 19-year warming experiment showed that warming significantly altered the stoichiometric nutrient demand of soil microorganisms and the enzyme ratio of N: P in subarctic tundra (Stark et al., 2018). However, it has also been shown that a temperature increase has no significant effect on EEAs in a warming manipulation experiment on the Tibetan Plateau (Jing et al., 2014). In addition, the changes of soil water availability due to warming can also affect EEAs by influencing the substrate diffusion rate of extracellular enzyme into the surrounding environment (Geisseler et al., 2011; Gomez et al., 2020). Therefore, it is necessary to understand how soil microbial metabolic activities respond to climate warming by examining changes in EEAs and ecoenzymatic stoichiometry, which can help to predict the effect of global warming on soil nutrient cycling.

Given that soil nutrients are generally concentrated at the surface of the soil, previous studies concerning patterns of soil EEAs under warming were most focused to surface soil (Hofmann et al., 2016; Zuo et al., 2018). Owing to the long turnover time and good insulation at depth, subsoil C is conventionally assumed to be relatively stable to global warming (Harrison et al., 2011). However, subsoil in high-altitude ecosystem is increasingly concerned under global warming because of the huge amounts of organic C pool (Yang et al., 2008). Climate warming will affect soils initially at the topsoil, and then gradually to subsoil through downward heat flow and changes in plant and microbial residues inputs (Ding et al., 2019; Fierer et al., 2003; Zhou et al., 2020). The change of resource availability caused by warming may have exacerbated effects on subsoil microbial communities, where microorganisms have strong resource demands due to the decrease in nutrient availabilities with depth (Dove et al., 2021). The emerging study showed substantial changes in C dynamics of the subsoil but not topsoil in the Qinghai-Tibetan alpine grasslands over 5 years of warming (Jia et al., 2019). Plant roots will grow deeper under warming-induced nutrient limitation or moisture, which may potentially increase root inputs and enhance C accumulation in the subsoil (Cotrufo et al., 2015; Liu et al., 2018). As EEAs is very sensitive to the changes of the soil environment, the effect of warming on the C pools of the topsoil and subsoil can be explored by using ecoenzymatic stoichiometry. Hence, investigation of microbial metabolic activity in different soil layers is essential to reveal nutrient cycling patterns and identify organic C pools that are sensitive to climate warming.

Altitudinal gradients can test the long-term effects of global warming on soil nutrient cycling via the changes in climatic and soil properties (Cao et al., 2021; Zhang et al., 2019). The effects of dramatic changes in the soil environment (e.g., temperature) over a short spatial distance may influence SOM decomposition, and then soil nutrient and microbial metabolic activity (Burns et al., 2013; Zuo et al., 2021). Numerous studies have shown that climatic factors and geographical variations may affect soil EEAs and their responses to climate warming (German et al., 2012; Meng et al., 2020). Cui et al. (2019a) revealed that the microbial C and P limitations were higher at the high elevation than the low elevation in alpine *Abies fabri* forest. Zuo et al. (2019) found that soil nutrient altering soil EEAs indirectly through affecting microbial biomass at low elevation, and soil moisture influenced soil EEAs via pH at the mid-elevation. Consequently, investigating the altitudinal pattern of EEAs will be helpful for identifying the key drivers on the dynamics of EEAs and microbial metabolic activities.

Here we set out a warming experiment in the Yajiageng alpine grassland from the eastern Tibetan Plateau to determine the effects of warming on soil microbial activity along the altitudinal gradients. We investigated microbial metabolic limitation in the topsoil and subsoil of alpine grassland in response to the experimental warming along an altitudinal gradient from 3800 to 4200 m. The main objectives of this study are to (1) determine the effect of warming on microbial metabolic limitation characteristics in topsoil and subsoil along the altitudinal gradient; (2) illuminate the dominant factor that affects microbial metabolic limitation in topsoil and subsoil of alpine grassland. We hypothesized that (1) microbial nutrient metabolic limitation increaseed with elevation, yet it might be alleviated under warming; and (2) microbial metabolic limitation in topsoil was more sensitive to warming than that in subsoil.

2. Materials and methods

2.1. Study area and experimental design

The study area is located at the Yajiageng (29°51′36″-29°52′48″ N, 102°1′12″-102°1′48″ E) on the eastern slope of Gongga Mountain (Fig. S1), which is located on the transition zone between the eastern subtropical humid monsoon zone and the cold climate of the Tibetan plateau, with a mean annual temperature of 4.1 °C and the mean annual precipitation of 1691 mm (Wang et al., 2012). The digital elevation model data on the Tibetan Plateau were obtained from Farr et al. (2007). This warming experiment was established in the alpine grassland in 2016 along four altitudinal gradients (3800, 4000, 4100 and 4200 m). The open-top chambers (OTCs; Fig. S1) were used to rise the temperature averaged by 1.5-2 °C at the four sites. The experiment plots were designed with no-warming (CK), and warming (W) with three replications.

2.2. Soil sampling and physicochemical analysis

We collected topsoil (0-5 cm) and subsoil (5-10 cm) samples (Fig. S1) from four altitudes at CK and W treatments in August 2018. The soils were passed through a 2-mm sieve in field after the visible roots were removed. Then each soil sample was divided into two subsamples. One subsample was immediately stored at 4 °C for the analysis of extracellular enzyme activities and microbial biomass. The other subsample was oven-dried with gentle air flow for physicochemical properties analysis.

Soil water content (SWC) was determined gravimetrically by over-drying 20 g of fresh soil at 105 °C overnight. Soil pH was estimated in a 1: 2.5 soil: water (*w/v*) mixture using a glass electrode meter (InsMark™ IS126, Shanghai, China). Soil organic C (SOC) was determined by a titration method based on the oxidation of organic substances with potassium dichromate external heating. The total nitrogen (TN) was measured using the Kjeldahl method using the Kjeltec 8400 (FOSS, Denmark). The total phosphorus (TP) was measured by an ultraviolet spectrophotometer (UV3200, Shimadu Corporation, Japan) after wet digestion with H_2SO_4 and $HClO_4$. Dissolved organic C (DOC) was extracted with 0.5 M K_2SO4 and shaken for 60 min, and then the extracts were measured using a Liqui TOCII analyzer (Elementar, Germany) after filtered through a Millipore 0.45-μm filter (Jones and Willett, 2006). Soil NH_4^+-N and NO_3^--N were determined the colorimetric method using an using an Auto Analyzer (SEAL Analytical Ltd., Norderstedt, Germany) after being extracted with 2 mol/L KCl. Olsen-P was measured using the 0.5 M $NaHCO_3$ extraction-ammonium molybdate-antimony potassium tartrate and ascorbic acid spectrophotometric method. Microbial biomass C (MBC), microbial biomass N (MBN) and microbial biomass P (MBP) were determined using the chloroform fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987), and the derived conversion factors were 0.45, 0.54 and 0.4 for MBC, MBN and MBP, respectively (Joergensen, 1996). The ratios of SOC to TN (C: N), SOC to TP (C: P) and TN to TP (N: P) were calculated.

2.3. Analyses of soil potential extracellular enzyme activities

The EEAs of two C-acquiring enzymes (β-1,4-glucosidase (BG) and β-D-cellobiosidase (CBH)), two N-acquiring enzymes (β-1,4-N-acetylglucosaminidase (NAG) and L-leucine aminopeptidase (LAP)), and one P-acquiring enzyme (acid phosphatase (AP)) were assayed by the method of Saiya-Cork et al. (2002). We conducted assays using 96-well plates per sample per assay. The potential EEAs were measured fluorometrically using a 200-μM solution of substrates labelled with 4-

methylumbelliferone (MUB). The samples of each assay included a blank, a negative control, and a quench standard. 1 g of fresh soil was homogenized in 125 mL 50mM buffer on a shaker at 25 °C for 2 h. Then, the microplates were incubated in the dark at 25 °C for 4 h. After incubation, 1 mL aliquot of 1M NaOH was added to each well to stop the reaction. Finally, the fluorescence was then measured using a microplate fluorometer with 365 nm excitation and 450 nm emission filters (German et al., 2011). Following correction of the fluorescence measurements of the assay wells for the negative controls, blanks, and quench standard wells, EEAs were expressed as nanomoles of substrate released per hour per gram of SOM (nmol g SOM^{-1} h^{-1}).

2.4. Microbial nutrient limitations

Equations (1-3) were used to calculate the stoichiometric of the EEAs. C: N_{EEA} represents the ratio of C- and N-acquiring enzymes; C: P_{EEA} represents the ratio of C- and P-acquiring enzymes; N: P_{EEA} represents the ratio of N- and P-acquiring enzymes.

$$C: N_{EEA} = \mathrm{Ln}\ (BG+CBH)/\mathrm{Ln}\ (LAP + NAG) \quad (1)$$

$$C: P_{EEA} = \mathrm{Ln}\ (BG+CBH)/\mathrm{Ln}\ AP \quad (2)$$

$$N: P_{EEA} = \mathrm{Ln}\ (LAP + NAG)/\mathrm{Ln}\ AP \quad (3)$$

We quantified microbial nutrient limitation by calculating the vector lengths and angles based on EEAs. Vector length and angle were calculated based on the equation proposed by Moorhead et al. (2016). Vector length, representing relative C or nutrient limitation, was calculated as the square root of the sum of x^2 and y^2, where x represents the relative activities of C- or P-acquiring enzymes and y represents the relative activities of C- or N-acquiring enzymes (Eq. 4). Vector angle, representing relative P or N limitation, was calculated as the arctangent of the line extending from the plot origin to point (x, y) (Eq. 5). Vector angle < 45° represents microbial N limitation, and vector angle > 45° represents P limitation. Microbial N limitation decreases with the vector angle, and microbial P limitation increases with the vector angle.

$$Length = SQRT\ (x^2 + y^2) \qquad (4)$$

$$Angle\ (°) = DEGREES\ (ATAN2\ (x, y)) \qquad (5)$$

2.5. Statistical analysis

All statistical calculations (i.e., correlations and significant differences) were conducted using the SPSS 20.0 statistical software package (SPSS Inc, Chicago, USA). Two-way analysis of variance (ANOVA) and least significant difference (LSD) multiple comparison ($P < 0.05$) were used to assess the significance of differences among the different sampling sites. The liner regression analysis and graphs were created using Origin 2021. A redundancy analysis (RDA) was used to examine vectors changed in relation to the soil factors using Canoco 5.0 software (Microcomputer Power, Inc., Ithaca, NY), and the significance of the factors was measured using Monte Carlo permutations test with 499 permutations. Partial least squares path modeling (PLS-PM) was used to further identify the possible pathways of various factors affecting microbial metabolic limitation. The models were constructed using the “innerplot” function of the R package “plspm”.

3. Results

3.1. Soil characteristics along the altitudinal gradient

The soil properties under warming along the elevation gradient are shown in Table 1. The lowest and highest values of SWC in topsoil were observed respectively at 4,200 m a.s.l and 4,000 m a.s.l, and it was significantly higher at 4,000 m a.s.l and 4,100 m a.s.l in subsoil. The SOC content was significantly lower at high altitude than that low altitude, and warming significantly increased SOC content at 4000m a.s.l ($P < 0.05$). Neither TN nor TP was significantly altered according to the elevation gradients or the warming treatment. DOC content in topsoil decreased progressively with altitude and increased significantly at 4100 m a.s.l due to warming ($P < 0.05$). Olsen-P in topsoil was significantly higher at lower altitudes than at higher altitudes, and warming resulted in significant increases in Olsen-P at 3800 and 4000 m a.s.l ($P < 0.05$). Warming significantly increased soil MBP content at 4000 m a.s.l in both topsoil and subsoil ($P < 0.05$).

3.2. Variation of soil extracellular enzyme activities and stoichiometry

Regardless of experimental warming, elevation had significant effects on EEAs in topsoil and subsoil ($P < 0.05$, Fig. S2). C-acquiring enzymes increased significantly with altitude gradient in both topsoil and subsoil. N-acquiring enzymes were significantly higher at 4000 m a.s.l than 3800 m a.s.l in both topsoil and subsoil ($P < 0.05$).The activities of P-acquiring enzymes were significantly higher at 4200 m a.s.l than those at other altitudes in topsoil,which were significantly lower at 3800 m a.s.l than those at other altitudes in subsoil ($P < 0.05$). Compared with altitude gradient, the effect of warming treatment on soil EEAs was limited. Warming had only a significant effect on C- and N-acquiring in topsoil, and elevation and warming had a significant interactive effect on EEAs in topsoil ($P < 0.05$, Fig. S2). Stoichiometric ratios of C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient were shown in Fig. 1. C: N_{EEA} increased with elevation both in topsoil and subsoil, and warming significantly decreased the C: N_{EEA} at 3800 and 4200 m a.s.l ($P < 0.05$). C: P_{EEA} is highest at 4100 m a.s.l in topsoil and at 4200 m in subsoil, and it was significantly reduced under the effect of warming at 4100 m a.s.l in both the topsoil and subsoil ($P < 0.05$). N: P_{EEA} was significantly lower at higher altitudes than at lower altitudes ($P < 0.05$). Warming significantly increased soil N: P_{EEA} at 3800 and 4200 m a.s.l, whereas it decreased soil N:P_{EEA} at 4100 and 4200 m a.s.l in topsoil ($P < 0.05$).

Altitude had a significant effect on the vector angles in both topsoil and subsoil ($P < 0.001$, Fig. 2). Altitude and warming had significant interactive effect on the vector lengths and angles in the topsoil. Vector lengths gradually increased with the altitude gradient in both topsoil and subsoil, and got the highest value at 4200 m a.s.l. Warming treatment had a significant effect on the vector length and significantly decreased the microbial C limitation of soil at 4100 m a.s.l in the topsoil ($P < 0.05$, Fig. 2A and B). All vector angles in both topsoil and subsoil indicate P limitation relative to N in the microbial community (Fig. 2C and D). Because of warming, microbial P limitation significantly increased at 4000 m a.s.l and 4100 m a.s.l in topsoil, and decreased at 3800 m a.s.l and 4200 m a.s.l in topsoil and 4200 m a.s.l in subsoil. In addition, the linear regression analysis showed a significantly negative correlation between vector length and angle in topsoil and subsoil along an altitudinal gradient ($P < 0.001$, $P < 0.05$, respectively, Fig. 3).

3.3. Relationships between extracellular enzyme activities and stoichiometry with soil variables

The results of linear regression analysis showed that microbial C limitation in topsoil was negatively correlated with SWC, SOC, TN, C: N ratio, DOC, NO_3^--N and NH_4^+-N, Olsen-P, and MBP, and positively with Mc:p; in subsoil, microbial C limitation was negatively correlated with C: P ratio and MBP, and positively with TP, DOC and Mc:p (Fig. S3). The microbial P limitation in topsoil was negatively correlated with C: P ratio and MBP, and positively with MBN; in subsoil, microbial P limitation was significantly correlated negatively with TP and Olsen-P, and positively with SWC, C: P ratio, MBC and MBN (Fig. S4).

Redundancy analysis (RDA) revealed that abiotic and biotic factors together accounted for 85% (RDA1, 59.5%; RDA2, 25.5%) of the total variation in microbial C and P limitation in topsoil, and explaining 93% (RDA1, 74.9%; RDA2, 16.4%) of these in subsoil (Fig. 4). To minimize the confounding interactions among causal factors, PLS-PM was implemented to further identify direct and indirect effects of soil physical properties, total and available nutrient contents, nutrient ratios, microbial biomass and their ratios on the microbial C and P limitations. The model indicated one of the best fit to the topsoil and subsoil data with goodness-of-fit of 61.3% and 53.9%, respectively (Fig. 5). In topsoil, total nutrient (-0.66), SWC (-0.45) and available nutrient (-0.4) had negative total effects on the microbial C limitations, whereas the nutrient ratios (0.53) and microbial biomass (0.36) induced positive total effects (Fig. 5A). Meanwhile, microbial biomass ratios (0.53), available nutrient (0.24) and total nutrient (0.14) had positive total effects on the microbial P limitations, whereas the microbial biomass (-1) induced the highest negative total effects. In subsoil, SWC (0.37), nutrient ratios (0.2) and microbial biomass ratios (0.2) had positive total effects on the microbial C limitations, whereas the microbial biomass (-0.5), available nutrient (-0.39) and total nutrient (-0.37) had negative total effects. Furthermore, the negative total effects of microbial biomass ratios (-0.66) were the highest for microbial P limitations in subsoil, whereas the available nutrient (0.12) induced the highest positive total effects (Fig. 5B).

4. Discussion

4.1. The patterns of nutrient limitation with warming along elevation gradient in alpine grasslands

Our results showed the patterns of microbial metabolic limitation warming-induced along elevation gradient in alpine grasslands. Firstly, microbial metabolism underwent relative C and P limitation in alpine grassland ecosystem, and C limitation increased significantly with the altitude gradient (Figs. 1 and 2). Soil environment, such as soil moisture and nutrient availability, plays a critical role in soil microbial activity. In general, soil temperatures decrease steadily with altitude increasing, but soil moisture and substrate do not always follow this specific trend (Cao et al., 2021; Tan et al., 2021). In this study, the low soil water content and nutrient availability are relatively low in high altitude area (Table 1), which suppress microbial metabolic activity and nutrient acquisition of microorganisms. Meanwhile, the lowest values of MBC and MBP were observed in high altitude, which could be due to microorganisms devoting more resources to enzyme production to obtain more nutrients based on resource allocation theory. Secondly, warming would alleviate microbial C metabolic limitation, especially at 4100 m a.s.l (Fig. 2). Zuccarini et al. (2020) indicated that warming could lead to faster mineralization of soil organic matter and water consumption in colder climates. The alleviation of microbial metabolic limitation might be due to the increase of EEAs catalytic ability and microbial metabolic activity for warming promoting the decomposition of organic matter. In this study, a significant increase in DOC content at 4100 and 4200 m a.s.l in the warming treatment confirmed this explanation (Table 1). Thirdly, compared with the topsoil, there were non-significant variations of EEAs and stoichiometry between warmed and control plots in subsoil (Figs. 1 and 2), meaning that nutrients cycling process in subsoil could not be greatly changed by warming in alpine grasslands. Due to good insulation at depth, the state of subsoil is generally considered to be relatively stable and insensitive to warming-induced (Harrison et al., 2011), which may explain that the EEAs and stoichiometry in subsoil had no obvious response to warming-induced.

4.2. Key factors drivering microbial metabolic characteristics of topsoil and subsoil in the alpine grassland

Nutrient content and nutrient ratio (SOC, Olsen-P, C: P) are the key factors affecting C limitation in alpine grassland. Concretely, the microbial C limitation was negatively correlated with SOC, TN, DOC, NO_3^--N and NH_4^+-N and Olsen-P in topsoil (Fig. S3), and the PLS-PM further identified that the total nutrient and available nutrient had the greater negative total effects than others (Fig. 5). The relative low nutrient contents such as DOC and Olsen-P may aggravate the relative C limitation of microorganisms, indicating that enzyme activities are limited by the availability of C and P. Previous studies have also demonstrated that the availability of nutrients is powerful controller of microbial processes including the metabolic activity of microorganisms (Cui et al., 2019b; Xiao et al., 2020). Meanwhile, it is interesting to note that C: P ratios was negatively correlated with the microbial C limitation in both the topsoil and subsoil, but positively correlated with the microbial P limitation in subsoil (Figs. S3 and S4). The result of PLS-PM further confirmed that nutrient ratio induced the greatest positive total effect on microbial C limitation. Specifically, relative high C: P ratios were observed at the low altitude corresponding to lower microbial C limitation, which is conductive to soil C sequestration. Our studies have pointed out that the variation of nutrient ratios due to the imbalance supply of soil nutrients can induce soil microbial metabolic limitation, even although the alpine ecosystem has relatively sufficient nutrient availability (Cui et al., 2019a). Differences in microbial abilities to acquisition C, N and P vary in soils with different nutrient states in order to maintain microbial homeostasis (Peng and Wang, 2016; Sinsabaugh and Shah, 2012). For instance, lower P: N_{EEA} ratios were related to the higher soil P availability compared with N availability in the drylands of northern China (Feng et al., 2019), and He et al. (2020) also found that the microbial nutrient status is the main factor modulating enzyme stoichiometry in subalpine forests. In our study, soil nutrients and their stoichiometric ratios are still important factors influencing the C metabolic limitation of soil microorganisms, even in alpine grasslands with high C and N content.

Our results also showed that the microbial biomass and its ratio are the pivotal factors to regulate the microbial P limitation in the alpine grassland ecosystem. The PLS-PM confirmed that microbial biomass had the greatest direct impact on microbial P limitations in both topsoil and subsoil (Fig. 5). As a big recharge source and reserve of soil available nutrient, MBP reflects the rate of P turnover in soil with the involvement of microorganisms and has been an important indicator for soil P availability (Ding et al., 2019). To cope with P deficiency in alpine grasslands, soil microorganisms invest relatively more P-acquiring enzymes than C- and N-acquiring enzymes. Interestingly, we found that the microbial C limitation was correlated negatively with MBP and positively with Mc:p in both topsoil and subsoil, but MBP and Mc:p didn't no significantly correlated with microbial P limitation (Fig. S3). It can be explained that microbial biomass and its ratio can affect the P limitation by influencing the microbial C limitation. Warming can increase the demand for C to meet the growth requirement of microorganisms and the demand for P correspondingly, which aggravate the relative P limitation of microorganisms. The PLS-PM further confirmed that microbial biomass ratio has the greatest total effect on the microbial P limitations (Figs. 4 and 5).

4.3. The potential implications of microbial metabolic limitations for soil C turnover in alpine grassland ecosystems

The ecological stoichiometric ratios of soil enzymes have been increasingly used to predict ecosystem stability and C cycle (Xu et al., 2017). Soil microbial metabolism related to soil enzyme production is regulated by soil biotic and abiotic factors, which in turn plays a vital role in soil C turnover in terrestrial ecosystems. The EEAs excreted by soil microorganisms are directly involved in SOM decomposition and nutrient turnover. Our study showed that decreased SOC and increased microbial C limitation with the altitude in topsoil (Figs. 4 and S3), and warming could relieve microbial C limitation (Fig. 2). Soil nutrients in our study decreased significantly with altitude, indicating that soil may provide more available nutrients with higher SOC content to meet microbial nutrient requirements, thereby relieving microbial C limitation. Warming may accelerate C decomposition and thus allocating more resources to soil microbial growth. As microbial C limitation decreasing, soil microbial communities may shift from maintenance respiration to growth and decrease investment in the production of enzymes to maintain homeostasis as organic matter decomposition progresses. Recent studies from regional to global scales have shown that C use efficiency increases with mean annual temperature (Sinsabaugh et al., 2017; Takriti et al., 2018), although rising soil temperatures are generally expected to reduce C use efficiency (Steinweg et al., 2008; Tucker et al., 2013). Microbial respiration rates may increase more with temperature than the rates of microbial growth. In addition, warming may be beneficial to increase the accumulation of microbial residues in SOC, which is of great ecological implications for the storage of soil C.

Simultaneously, it is worthy to note that there are intrinsic linkages of biological C and P acquisitions and turnover. P sources required by microorganisms mostly come from soil organic matter decomposition, hence microbial demand for P will strongly affect soil C stability (Cui et al., 2020). There was a significant negative correlation between C and P limitation in topsoil and subsoil (Fig. 3), implying the coupling between microbial C and P limitations. Specifically, our results observed that warming resulted in not only a decrease in the C: N_{EEA} ratio but also an increase in the N: P_{EEA} ratios at 3800 and 4200 m a.s.l. This phenomenon can be explained by the growth rate hypothesis that microbial growth rates are controlled by the cellular P quota (Allen and Gillooly, 2009; Frost et al., 2006). As a rock-derived element, P is released mainly through geochemical weathering and is difficult to obtain from atmosphere. In the alpine grassland ecosystem, unfortunately, high precipitation and soil moisture accelerate the leaching of soil P (He et al., 2018; Manzoni et al., 2010), making soil P more deficient for plant growth and soil microorganism decomposition. The increase of soil microbial P demand will accelerate the decomposition of SOM to obtain P, which will have an important impact on the soil C pool. Consequently, the incorporation of microbial metabolic limitations into global C cycle models will improve the accuracy of soil C sequestration estimation in the context of global climate change.

5. Conclusions

Our results revealed that microbial C limitation increased with elevation in both topsoil and subsoil in alpine grassland. Warming relieved microbial C limitation in topsoil, suggesting that global warming may be beneficial to soil C sequestration in the alpine ecosystems. Furthermore, the key factors affecting C limitation were nutrients content and nutrient stoichiometry, and the microbial P limitation was mainly regulated by microbial biomass and their ratios. The study provides insight into the mechanisms understanding of microbial metabolism mediation of C cycles, which is crucial for predicting future changes in nutrient cycling in alpine grassland ecosystem.

Acknowledgements

This study was financially supported by the Strategic Priority Research Program of Chinese Academy of Sciences (XDB40000000), the National Natural Science Foundation of China (41977031), and State Key Laboratory of Loess and Quaternary Geology, Institute of Earth Environment, CAS (SKLLQGZR1803).

Supplemental information

Supplementary information includes one table and three additional figures that topsoil and subsoil microbial biomass with warming in the alpine grassland along the elevation gradient (Table S1), study area and photos of the OTC warming experiment (Fig. S1), extracellular enzyme activity for C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient (Fig. S2), and vector length and angle in relation to soil physical properties, total nutrient contents, nutrient ratios, available nutrient contents, microbial biomass, and microbial biomass ratios (Figs. S3 and S4).

References

Allen, A.P., and Gillooly, J.F., 2009. Towards an integration of ecological stoichiometry and the metabolic theory of ecology to better understand nutrient cycling. Ecol. Lett. 12, 369-384.

Bardgett, R.D., Freeman, C., and Ostle, N.J., 2008. Microbial contributions to climate change through carbon cycle feedbacks. ISME J. 2, 805-814.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., and Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biol. Biochem. 58, 216-234.

Cao, R., Yang, W., Chang, C., Wang, Z., Wang, Q., Li, H., Tan, B., 2021. Differential seasonal changes in soil enzyme activity along an altitudinal gradient in an alpine-gorge region. Appl. Soil Ecol. 166.

Chen, H., Li, D., Xiao, K., and Wang, K., 2018. Soil microbial processes and resource limitation in karst and non-karst forests. Funct. Ecol. 32, 1400-1409.

Chen, L., Liang, J., Qin, S., Liu, L., Fang, K., Xu, Y., Ding, J., Li, F., Luo, Y., and Yang, Y., 2016. Determinants of carbon release from the active layer and permafrost deposits on the Tibetan Plateau. Nat. Commun. 7.

Crowther, T.W., Todd-Brown, K.E.O., Rowe, C.W., Wieder, W.R., Carey, J.C., Machmuller, M.B., Snoek, B.L., Fang, S., Zhou, G., Allison, S.D., Blair, J.M., Bridgham, S.D., Burton, A.J., Carrillo, Y., Reich, P.B., Clark, J.S., Classen, A.T., Dijkstra, F.A., Elberling, B., Emmett, B.A., Estiarte, M., Frey, S.D., Guo, J., Harte, J., Jiang, L., Johnson, B.R., Kroel-Dulay, G., Larsen, K.S., Laudon, H., Lavallee, J.M., Luo, Y., Lupascu, M., Ma, L.N., Marhan, S., Michelsen, A., Mohan, J., Niu, S., Pendall, E., Penuelas, J., Pfeifer-Meister, L., Poll, C., Reinsch, S., Reynolds, L.L., Schmidt, I.K., Sistla, S., Sokol, N.W., Templer, P.H., Treseder, K.K., Welker, J.M., and Bradford, M.A., 2016. Nature 540, 104-+.

Cui Y., Bing H., Fang L., Jiang M., Shen G., Yu J., Wang X., Zhu H., Wu Y., Zhang X., 2019. Extracellular enzyme stoichiometry reveals the carbon and phosphorus limitations of microbial metabolisms in the rhizosphere and bulk soils in alpine ecosystems. Plant Soil 458, 7-20.

Cui, Y., Fang, L., Deng, L., Guo, X., Han, F., Ju, W., Wang, X., Chen, H., Tan, W., and Zhang, X., 2019. Patterns of soil microbial nutrient limitations and their roles in the variation of soil organic carbon across a precipitation gradient in an arid and semi-arid region. Sci. Total Environ. 658, 1440-1451.

Cui, Y., Wang, X., Zhang, X., Ju, W., Duan, C., Guo, X., Wang, Y., and Fang, L., 2020. Soil moisture mediates microbial carbon and phosphorus metabolism during vegetation succession in a semiarid region. Soil Biol. Biochem. 147.

Ding, X., Chen, S., Zhang, B., Liang, C., He, H., and Horwath, W. R., 2019. Warming increases microbial residue contribution to soil organic carbon in an alpine meadow. Soil Biol. Biochem. 135, 13-19.

Dove, N.C., Torn, M.S., Hart, S.C., Tas, N., 2021. Metabolic capabilities mute positive response to direct and indirect impacts of warming throughout the soil profile. Nat. Commun. 12, 2089-2089.

Farr, T.G., Rosen, P.A., Caro, E., Crippen, R., Duren, R., Hensley, S., Kobrick, M., Paller, M., Rodriguez, E., Roth, L., Seal, D., Shaffer, S., Shimada, J., Umland, J., Werner, M., Oskin, M., Burbank, D., Alsdorf, D., 2007. The Shuttle Radar Topography Mission. Rev. Geophys. 45.

Feng, J., Wei, K., Chen, Z., Lu, X., Tian, J., Wang, C., and Chen, L., 2019. Coupling and decoupling of soil carbon and nutrient cycles across an aridity gradient in the drylands of northern China: evidence from ecoenzymatic stoichiometry. Glob. Biogeochem. Cycles 33, 559-569.

Fierer, N., Schimel, J.P., and Holden, P.A., 2003. Variations in microbial community composition through two soil depth profiles. Soil Biol. Biochem. 35, 167-176.

Frost, P.C., Benstead, J.P., Cross, W.F., Hillebrand, H., Larson, J.H., Xenopoulos, M.A., and Yoshida, T., 2006. Threshold elemental ratios of carbon and phosphorus in aquatic consumers. Ecol. Lett. 9, 774-779.

Geisseler, D., Horwath, W.R., Scow, K.M., 2011. Soil moisture and plant residue addition interact in their effect on extracellular enzyme activity. Pedobiologia 54, 71-78.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., and Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387-1397.

German, D.P., Marcelo, K.R.B., Stone, M.M., Allison, S.D., 2012. The Michaelis-Menten kinetics of soil extracellular enzymes in response to temperature: a cross-latitudinal study. Glob. Chang. Biol. 18, 1468-1479.

Gomez, E.J., Delgado, J.A., Gonzalez, J.M., 2020. Environmental factors affect the response of microbial extracellular enzyme activity in soils when determined as a function of water availability and temperature. Ecol. Evol. 10, 10105-10115.

Guan, P., Yang, J., Yang, Y., Wang, W., Zhang, P., Wu, D., 2020. Land conversion from cropland to grassland alleviates climate warming effects on nutrient limitation: Evidence from soil enzymatic activity and stoichiometry. Glob. Ecol. Conserv. 24.

Harrison, R.B., Footen, P.W., and Strahm, B.D., 2011. Deep soil horizons: contribution and importance to soil carbon pools and in assessing whole-ecosystem response to management and global change. For. Sci. 57, 67-76.

He, Q., Wu, Y., Bing, H., Zhou, J., and Wang, J., 2020. Vegetation type rather than climate modulates the variation in soil enzyme activities and stoichiometry in subalpine forests in the eastern Tibetan Plateau. Geoderma 374.

He, X., Zhou, J., Wu, Y., Bing, H., Sun, H., and Wang, J., 2018. Leaching disturbed the altitudinal distribution of soil organic phosphorus in subalpine coniferous forests on Mt. Gongga, SW China. Geoderma 326, 144-155.

Hofmann, K., Lamprecht, A., Pauli, H., and Illmer, P., 2016. Distribution of prokaryotic abundance and microbial nutrient cycling across a high-alpine altitudinal gradient in the Austrian Central Alps is affected by vegetation, temperature, and soil nutrients. Microb. Ecol. 72, 704-716.

Hu, Y., Jiang, L., Wang, S., Zhang, Z., Luo, C., Bao, X., Niu, H., Xu, G., Duan, J., Zhu, X., Cui, S., and Du, M., 2016. The temperature sensitivity of ecosystem respiration to climate change in an alpine meadow on the Tibet plateau: A reciprocal translocation experiment. Agric. For. Meteorol. 216, 93-104.

Jansson, J. K., and Tas, N., 2014. The microbial ecology of permafrost. Nat. Rev. Microbiol. 12, 414-425.

Jing, X., Wang, Y., Chung, H., Mi, Z., Wang, S., Zeng, H., and He, J.S., 2014. No temperature acclimation of soil extracellular enzymes to experimental warming in an alpine grassland ecosystem on the Tibetan Plateau. Biogeochemistry 117, 39-54.

Joergensen, R. G., 1996. The fumigation-extraction method to estimate soil microbial biomass: Calibration of the k (EC) value. Soil Biol. Biochem. 28, 25-31.

Jones, D. L., and Willett, V. B., 2006. Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. Soil Biol. Biochem. 38, 991-999.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. Glob. Biogeochem. Cycles 21, 11.

Ladwig, L.M., Sinsabaugh, R.L., Collins, S.L., and Thomey, M.L., 2015. Soil enzyme responses to varying rainfall regimes in Chihuahuan Desert soils. Ecosphere 6.

Liu, H., Mi, Z., Lin, L., Wang, Y., Zhang, Z., Zhang, F., He, J.S., 2018. Shifting plant species composition in response to climate change stabilizes grassland primary production. Proc. Natl. Acad. Sci. U.S.A. 115, 4051-4056.

Lu, M., Zhou, X., Yang, Q., Li, H., Luo, Y., Fang, C., Chen, J., Yang, X., and Li, B.,

2013. Responses of ecosystem carbon cycle to experimental warming: a meta-analysis. Ecology 94, 726-738.

Lv W., Luo C., Zhang L., Niu H., Zhang Z., Wang S., Wang Y., Jiang L., Wang Y., He J., Kardol P., Wang Q., Li B., Liu P., Dorji T., Zhou H., Zhao X., Zhao L., 2020. Net neutral carbon responses to warming and grazing in alpine grassland ecosystems. Agric. For. Meteorol. 280, 0168-1923.

Manzoni, S., 2017. Flexible carbon-use efficiency across litter types and during decomposition partly compensates nutrient imbalances-results from analytical stoichiometric models. Front Microbiol. 8.

Manzoni, S., Trofymow, J. A., Jackson, R. B., and Porporato, A., 2010. Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. Ecol. Monogr. 80, 89-106.

Meng, C., Tian, D.S., Zeng, H., Li, Z.L., Chen, H.Y.H., Niu, S.L., 2020. Global meta-analysis on the responses of soil extracellular enzyme activities to warming. Sci. Total Environ. 705, 9.

Moorhead, D.L., Rinkes, Z.L., Sinsabaugh, R.L., and Weintraub, M.N., 2013. Dynamic relationships between microbial biomass, respiration, inorganic nutrients and enzyme activities: informing enzyme-based decomposition models. Front Microbiol. 4.

Peng, X., and Wang, W., 2016. Stoichiometry of soil extracellular enzyme activity along a climatic transect in temperate grasslands of northern China. Soil Biol. Biochem. 98, 74-84.

Rosinger, C., Rousk, J., and Sanden, H., 2019. Can enzymatic stoichiometry be used to determine growth-limiting nutrients for microorganisms? - A critical assessment in two subtropical soils. Soil Biol. Biochem. 128, 115-126.

Saiya-Cork, K.R., Sinsabaugh, R.L., and Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biol. Biochem. 34, 1309-1315.

Sinsabaugh, R.L., Hill, B.H., and Shah, J.J.F., 2009. Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462, 795-U117.

Sinsabaugh, R.L., Moorhead, D.L., Xu, X., and Litvak, M.E., 2017. Plant, microbial and ecosystem carbon use efficiencies interact to stabilize microbial growth as a fraction of gross primary production. New Phytol. 214, 1518-1526.

Sinsabaugh, R.L., and Shah, J.J.F., 2012. Ecoenzymatic stoichiometry and ecological theory. Annu. Rev. Ecol. Evol. Syst. 43(1), 313-343.

Takriti, M., Wild, B., Schnecker, J., Mooshammer, M., Knoltsch, A., Lashchinskiy, N., Eloy Alves, R.J., Gentsch, N., Gittel, A., Mikutta, R., Wanek, W., and Richter, A., 2018. Soil organic matter quality exerts a stronger control than stoichiometry on microbial substrate use efficiency along a latitudinal transect. Soil Biol. Biochem. 121, 212-220.

Tan, B., Yin, R., Zhang, J., Xu, Z., Liu, Y., He, S., Zhang, L., Li, H., Wang, L., Liu, S., You, C., Peng, C., 2021. Temperature and moisture modulate the contribution of soil fauna to litter decomposition via different pathways. Ecosystems 24, 1142-1156.

Tapia-Torres, Y., Elser, J.J., Souza, V., and Garcia-Oliva, F., 2015. Ecoenzymatic stoichiometry at the extremes: How microbes cope in an ultra-oligotrophic desert soil. Soil Biol. Biochem. 87, 34-42.

Tucker, C.L., Bell, J., Pendall, E., and Ogle, K., 2013. Does declining carbon-use efficiency explain thermal acclimation of soil respiration with warming? Glob. Chang. Biol. 19, 252-263.

Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Glob. Chang. Biol. 15, 1631-1639.

Wang, J., Pan, B., Zhang, G., Cui, H., Cao, B., Geng, H., 2012. Late Quaternary glacial chronology on the eastern slope of Gongga Mountain, eastern Tibetan Plateau, China. Sci. China Earth Sci. 56, 354-365.

Xiao, L., Liu, G., Li, P., Li, Q., and Xue, S., 2020. Ecoenzymatic stoichiometry and microbial nutrient limitation during secondary succession of natural grassland on the Loess Plateau, China. Soil Tillage Res. 200.

Xiao, W., Chen, X., Jing, X., and Zhu, B., 2018. A meta-analysis of soil extracellular enzyme activities in response to global change. Soil Biol. Biochem. 123, 21-32.

Xu, Z., Yu, G., Zhang, X., He, N., Wang, Q., Wang, S., Wang, R., Zhao, N., Jia, Y., Wang, C., 2017. Soil enzyme activity and stoichiometry in forest ecosystems along the North-South Transect in eastern China (NSTEC). Soil Biol. Biochem. 104, 152-163.

Yang, Y., Fang, J., Tang, Y., Ji, C., Zheng, C., He, J., Zhu, B., 2008. Storage, patterns and controls of soil organic carbon in the Tibetan grasslands. Glob. Chang. Biol. 14(7), 1592-1599.

Zak, D. R., Pregitzer, K. S., Burton, A. J., Edwards, I. P., and Kellner, H., 2011. Microbial responses to a changing environment: implications for the future functioning of terrestrial ecosystems. Fungal Ecol. 4, 386-395.

Zhang, X., Shen, Z., Fu, G., 2015. A meta-analysis of the effects of experimental warming on soil carbon and nitrogen dynamics on the Tibetan Plateau. Appl. Soil Ecol. 87, 32-38.

Zhang, Y., Li, C., and Wang, M., 2019. Linkages of C: N: P stoichiometry between soil and leaf and their response to climatic factors along altitudinal gradients. J. Soils Sediments 19, 1820-1829.

Zheng, H., Liu, Y., Chen, Y., Zhang, J., Li, H., Wang, L., Chen, Q., 2020. Short-term warming shifts microbial nutrient limitation without changing the bacterial community structure in an alpine timberline of the eastern Tibetan Plateau. Geoderma 360.

Zhou, J., Deng, Y., Shen, L., Wen, C., Yan, Q., Ning, D., Qin, Y., Xue, K., Wu, L., He, Z., Voordeckers, J. W., Van Nostrand, J. D., Buzzard, V., Michaletz, S. T., Enquist, B. J., Weiser, M. D., Kaspari, M., Waide, R., Yang, Y., and Brown, J. H., 2016. Temperature mediates continental-scale diversity of microbes in forest soils. Nat. Commun. 7.

Zhou, L., Liu, S., Shen, H., Zhao, M., Xu, L., Xing, A., and Fang, J., 2020. Soil extracellular enzyme activity and stoichiometry in China's forests. Funct. Ecol. 34, 1461-1471.

Zhou, X., Chen, C., Wang, Y., Xu, Z., Duan, J., Hao, Y., Smaill, S., 2013. Soil extractable carbon and nitrogen, microbial biomass and microbial metabolic activity in response to warming and increased precipitation in a semiarid Inner Mongolian grassland. Geoderma 206, 24-31.

Zuccarini, P., Asensio, D., Ogaya, R., Sardans, J., and Penuelas, J., 2020. Effects of seasonal and decadal warming on soil enzymatic activity in a P-deficient Mediterranean shrubland. Glob. Chang. Biol. 26, 3698-3714.

Zuo, Y., Li, J., Zeng, H., and Wang, W., 2018. Vertical pattern and its driving factors in soil extracellular enzyme activity and stoichiometry along mountain grassland belts. Biogeochemistry 141, 23-39.

Zuo, Y., Zhang, H., Li, J., Yao, X., Chen, X., Zeng, H., Wang, W., 2021. The effect of soil depth on temperature sensitivity of extracellular enzyme activity decreased with elevation: evidence from mountain grassland belts. Sci. Total Environ. 777.

Figure captions:

Fig. 1 Stoichiometric ratios of C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient.

Fig. 2 The variation of vector length and angle in warmed plots (W) and non-warmed plots (CK) in different elevation gradients.

Fig. 3 Linear-regression analysis of microbial C limitation with microbial P limitation.

Fig. 4 Redundancy analysis (RDA) on soil enzymes activities and stoichiometry with climatic and soil factors as constraints in topsoil (A) and subsoil (B).

Fig. 5 Cascading relationships between microbial nutrient limitation with the soil properties, microbial biomass and their ratio.

Table 1 Topsoil and subsoil properties with warming in the alpine grassland along the elevation gradient.

	Altitude	Treats	SWC (%)	pH	SOC (g kg $^{-1}$)	TN (g kg $^{-1}$)	TP (g kg $^{-1}$)	DOC (mg kg $^{-1}$)	NO_3^--N (mg kg $^{-1}$)	NH_4^+-N (mg kg $^{-1}$)	Olsen-P (mg kg $^{-1}$)
0-5 cm	3800	W	0.44 ± 0.03 Aa	5.60 ± 0.16 Aa	148.31 ± 3.7 Ab	11.92 ± 1.25 Aa	1.01 ± 0.07 Aab	200.12 ± 12.7 Ab	203.75 ± 39.19 Aa	31.31 ± 4.2 Ab	6.58 ± 0.47 Aa
		CK	0.46 ± 0.03 Aab	5.12 ± 0.07 Aa	141.89 ± 5.15 Aa	10.6 ± 0.85 Aa	1.06 ± 0.04 Aa	186.71 ± 12.35 Aa	137.56 ± 10.12 Aa	44.89 ± 5.31 Aa	4.26 ± 0.32 Ba
	4000	W	0.52 ± 0.03 Aa	4.67 ± 0.2 Ab	174.6 ± 11.41 Aa	8.87 ± 0.67 Ab	0.81 ± 0.05 Ab	228.75 ± 6.72 Aa	151.09 ± 14.6 Aab	57.98 ± 3.09 Aa	4.17 ± 0.28 Ab
		CK	0.54 ± 0.06 Aa	4.60 ± 0.05 Ab	110.37 ± 1.58 Bb	8.91 ± 1.65 Aa	0.73 ± 0.07 Aa	180.95 ± 19.17 Aa	89.64 ± 3.0 Bb	32.09 ± 6.1 Bab	2.59 ± 0.2 Bbc
	4100	W	0.45 ± 0.01 Aa	5.23 ± 0.03 Aa	103.08 ± 1.62 Ac	7.59 ± 0.15 Ab	0.78 ± 0.1 Ab	211.81 ± 3.73 Aab	103.0 ± 6.33 Ab	17.8 ± 2.26 Bc	1.99 ± 0.1 Ac
		CK	0.42 ± 0.01 Ab	5.12 ± 0.02 Ba	101.8 ± 2.23 Ab	7.5 ± 0.32 Aa	0.86 ± 0.02 Aa	158.09 ± 7.7 Bab	65.28 ± 8.22 Bb	28.13 ± 2.57 Ab	2.21 ± 0.23 Ac
	4200	W	0.32 ± 0.03 Ab	5.29 ± 0.02 Aa	102.93 ± 7.25 Ac	7.91 ± 0.73 Ab	1.11 ± 0.08 Aa	194.79 ± 8.29 Ab	122.09 ± 13.6 Ab	32.18 ± 2.43 Ab	3.82 ± 0.23 Ab
		CK	0.35 ± 0.03 Ab	5.26 ± 0.06 Aa	108.71 ± 5.2 Ab	8.25 ± 0.36 Aa	1.03 ± 0.06 Aa	130.54 ± 7.18 Bb	107.19 ± 12.9 Aab	23.61 ± 2.66 Ab	3.34 ± 0.34 Ab
5-10 cm	3800	W	0.21 ± 0.01 Bb	5.49 ± 0.12 Aa	48.78 ± 2.81 Ab	5.21 ± 1.11 Aa	0.83 ± 0.14 Aab	67.97 ± 3.31 Ac	38.94 ± 1.71 Aa	6.77 ± 0.79 Bbc	1.66 ± 0.01 Ab
		CK	0.27 ± 0.02 Aa	5.08 ± 0.05 Ba	66.82 ± 6.75 Aa	4.81 ± 0.42 Aa	0.72 ± 0.02 Abc	61.08 ± 3.92 Ac	36.13 ± 1.4 Aa	10.15 ± 0.9 Aab	2.36 ± 0.4 Aab
	4000	W	0.4 ± 0.03 Aa	4.51 ± 0.12 Ac	73.03 ± 5.41 Aa	6.45 ± 2.33 Aa	0.62 ± 0.08 Ab	85.58 ± 4.76 Ab	21.09 ± 1.25 Ac	14.45 ± 1.08 Aa	1.84 ± 0.3 Aab
		CK	0.38 ± 0.04 Aa	4.51 ± 0.03 Ab	68.72 ± 8.69 Aa	4.65 ± 0.45 Aa	0.62 ± 0.01 Ac	70.37 ± 2.37 Bc	20.03 ± 4.3 Ab	11.14 ± 1.44 Aa	1.81 ± 0.14 Ab
	4100	W	0.39 ± 0.01 Aa	5.1 ± 0.03 Ab	64.94 ± 2.76 Aa	4.86 ± 0.24 Aa	0.75 ± 0.07 Aab	142.46 ± 4.3 Aa	29.64 ± 3.01 Ab	10.85 ± 2.5 Aab	1.54 ± 0.07 Ab
		CK	0.39 ± 0.01 Aa	4.99 ± 0.03 Ba	67.47 ± 1.87 Aa	5.0 ± 0.07 Aa	0.75 ± 0.01 Ab	125.86 ± 0.85 Ba	30.49 ± 3.76 Aab	14.61 ± 1.79 Aa	1.82 ± 0.13 Ab
	4200	W	0.25 ± 0.02 Ab	5.11 ± 0.06 Ab	64.35 ± 1.06 Aa	4.95 ± 0.3 Aa	0.99 ± 0.02 Aa	81.96 ± 7.14 Abc	29.31 ± 0.68 Ab	4.21 ± 1.19 Ac	2.32 ± 0.2 Aa
		CK	0.25 ± 0.03 Aa	5.07 ± 0.08 Aa	58.96 ± 2.58 Aa	5.14 ± 0.27 Aa	0.89 ± 0.07 Aa	92.04 ± 7.12 Ab	36.56 ± 5.86 Aa	6.49 ± 1.3 Ab	3.12 ± 0.31 Aa

Note: Different uppercase letters indicate significant differences ($P < 0.05$) between the W and CK treatments at each altitude, and different lowercase letters indicate significant differences ($P < 0.05$) amongst the altitudes in the W or CK treatment. SWC, soil water content; SOC, soil organic C; TN, soil total N; TP, soil total P; DOC, soil dissolved organic C. Data are presented as mean ± standard error (n = 3).

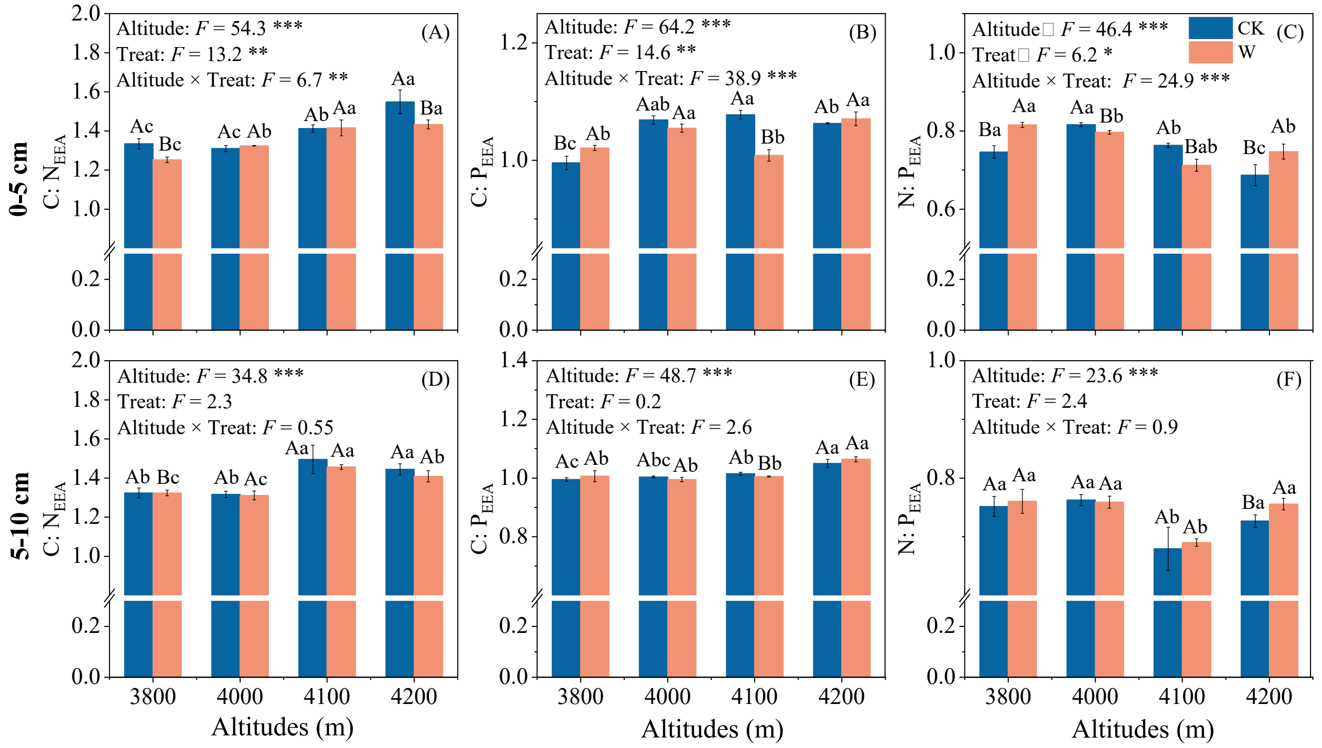

Fig. 1 Stoichiometric ratios of C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient. Error bars indicate standard error of the treatment mean (n=3). Different uppercase letters indicate significant differences ($P < 0.05$) between the W and CK treatments at each altitude, and different lowercase letters indicate significant differences ($P < 0.05$) amongst the altitudes in the W or CK treatment. C-acquiring enzymes, β-1,4-glucosidase (BG) and β-D-cellobiosidase (CBH); N-acquiring enzymes, β-1,4-N-acetylglucosaminidase (NAG) and L-leucine aminopeptidase (LAP); P-acquiring enzyme, acid phosphatase (AP). C: N_{EEA}: the ratio of C-acquiring enzymes to N-acquiring enzymes; C: P_{EEA}: the ratio of C-acquiring enzymes to P-acquiring enzymes; N: P_{EEA}: the ratio of N-acquiring enzymes to P-acquiring enzymes; Two-way ANOVA was used to test the effects of warming experiment, elevation gradient and their interactions.

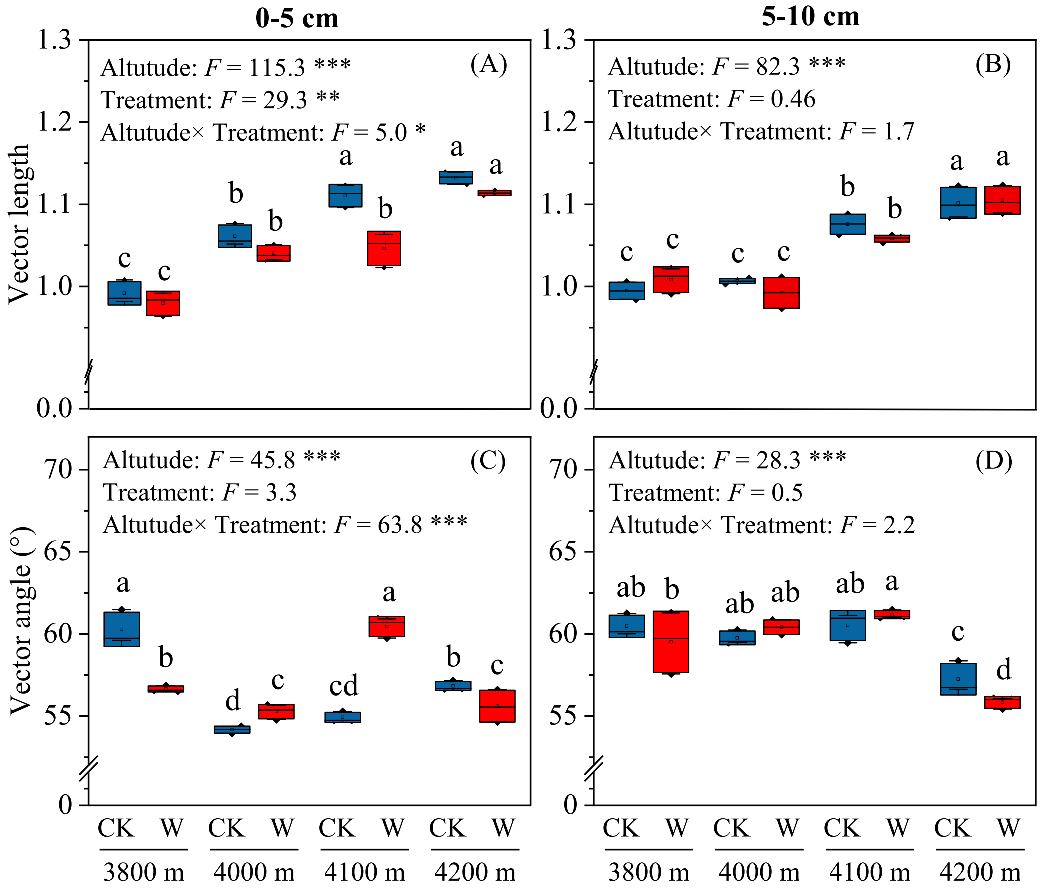

Fig. 2 The variation of vector length and angle in warmed plots (W) and non-warmed plots (CK) in different elevation gradients. Different letters indicate significant differences ($P < 0.05$) amongst the altitudes in the W and CK treatments. Vector length represents soil C limitation for microbes, vector angle represents soil N/P limitation for microbes.

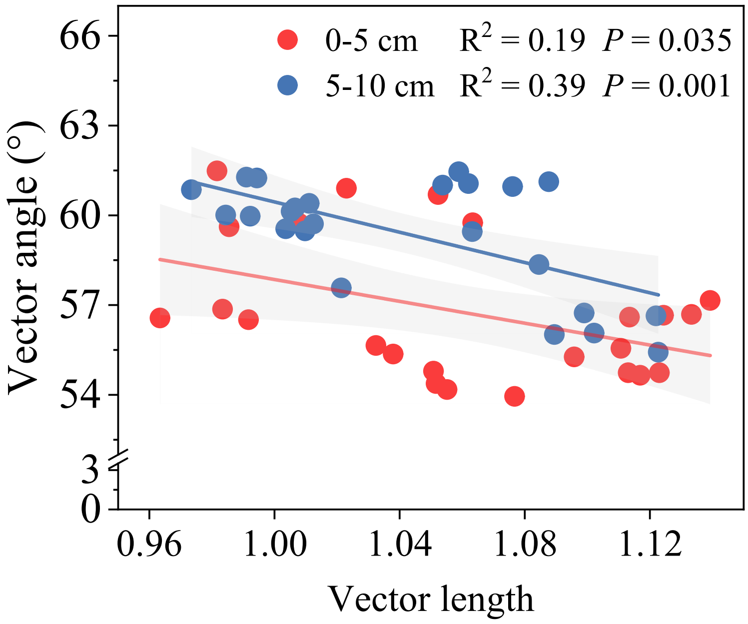

Fig. 3 Linear-regression analysis of microbial C limitation with microbial N/P limitation. Solid lines indicate the model fits between the vector lengths and the properties, and grey areas are the 95% confidence intervals of the models.

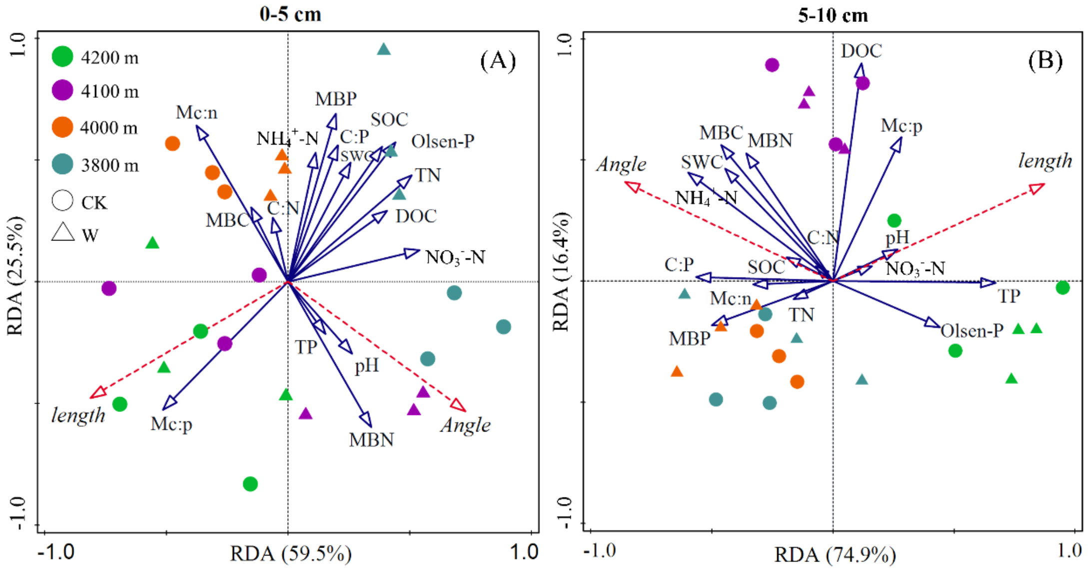

Fig. 4 Redundancy analysis (RDA) on soil enzymes activities and stoichiometry with climatic and soil factors as constraints in topsoil (A) and subsoil (B). A type III scaling (correlation) plot is shown, including all the constraining variables. The constraining variables explained 85.0 % and 91.3 % of the total variance of microbial metabolism limitations in the topsoil and subsoil, respectively. A taxonomic group arrow pointing in the same direction means a stronger positive correlation, and a longer arrow represents a stronger relationship.

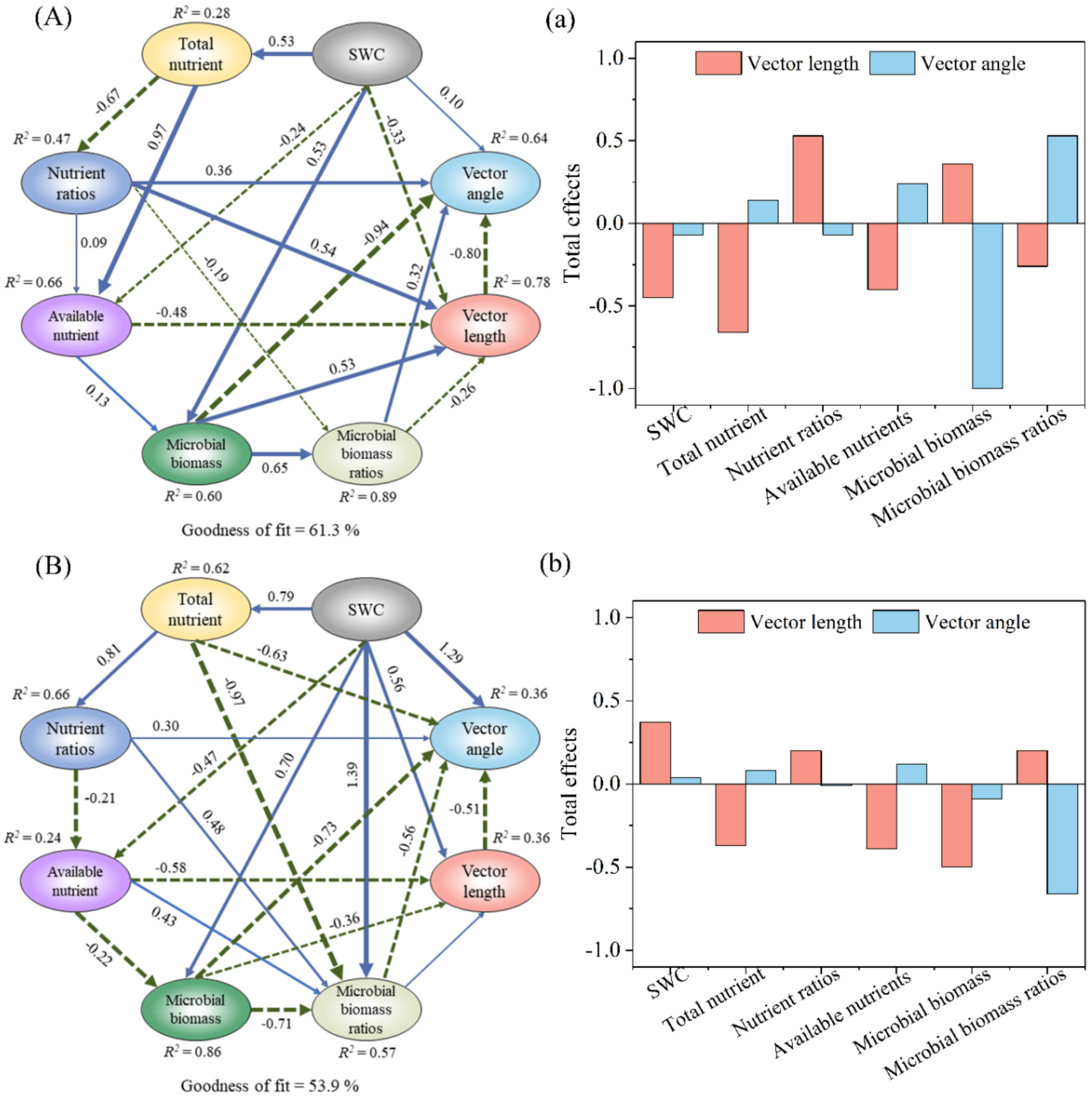

Fig. 5 Cascading relationships between microbial nutrient limitation with the soil properties, microbial biomass and their ratio. Partial least squares path modeling (PLS-PM) disentangling major pathways of the influences of soil water content (SWC), soil total nutrient (SOC, TN, TP), nutrient ratios (C: N, C: P), available nutrient (DOC, NO_3^--N, NH_4^+-N, Olsen-P), microbial biomass (MBC, MBN, MBP), microbial biomass ratios ($M_{C:P}$, $M_{C:N}$) on microbial C limitation (represented by vector length), microbial P limitation (represented by vector angle) in topsoil soil (A and a) and subsoil (B and b). Blue and Green arrows indicate positive and negative flows of causality ($P < 0.05$), respectively.

Supporting information

Table S1 Topsoil and subsoil microbial biomass with warming in the alpine grassland along the elevation gradient.

Fig. S1 Study areaand photos of the OTC (open-top chamber) warming experiment in an alpine grassland at the eastern edge of the Tibetan Plateau.

Fig. S2 Extracellular enzyme activity for C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient.

Fig. S3 Vector length in relation to soil physical properties, total nutrient contents, nutrient ratios, available nutrient contents, microbial biomass, and microbial biomass ratios.

Fig. S4 Vector angle in relation to in relation to soil physical properties, total nutrient contents, nutrient ratios, available nutrient contents, microbial biomass, and microbial biomass ratios.

Table S1 Topsoil and subsoil microbial biomass with warming in the alpine grassland along the elevation gradient.

	Altitude	Treats	MBC (mg kg $^{-1}$)	MBN (mg kg $^{-1}$)	MBP (mg kg $^{-1}$)
0-5 cm	3800	W	1498.66 ± 87.35 Ab	54.99 ± 4.61 Ab	58.47 ± 8.98 Ab
		CK	1377.92 ± 33.48 Ab	53.78 ± 2.89 Aa	44.88 ± 3.51 Ab
	4000	W	2223.75 ± 77.15 Aa	44.71 ± 3.42 Ab	108.54 ± 10.51 Aa
		CK	2123.52 ± 67.09 Aa	48.12 ± 1.81 Aa	66.2 ± 9.83 Ba
	4100	W	2089.83 ± 101.59 Aa	82.96 ± 2.21 Aa	30.89 ± 3.47 Ac
		CK	2050.65 ± 48.19 Aa	58.86 ± 5.37 Ba	20.71 ± 2.12 Ac
	4200	W	1499.73 ± 84.27 Ab	48.19 ± 1.8 Ab	13.92 ± 1.09 Ac
		CK	1264.45 ± 27.96 Ab	57.84 ± 11.11 Aa	22.18 ± 3.16 Ac
5-10 cm	3800	W	445.04 ± 15.34 Ab	23.45 ± 1.58 Ab	4.02 ± 0.39 Bc
		CK	519.65 ± 51.82 Ab	20.78 ± 4.35 Ac	9.24 ± 0.41 Ab
	4000	W	1108.31 ± 68.01 Aa	50.6 ± 12.64 Aa	28.8 ± 2.81 Aa
		CK	1048.34 ± 69.08 Aa	35.92 ± 3.17 Ab	16.56 ± 2.87 Ba
	4100	W	1103.13 ± 54.29 Aa	49.56 ± 6.6 Aa	11.91 ± 2.52 Ab
		CK	1146.87 ± 89.25 Aa	51.4 ± 3.63 Aa	7.2 ± 1.91 Abc
	4200	W	465.18 ± 25.34 Ab	23.86 ± 1.21 Ab	8.75 ± 1.54 Abc
		CK	455.86 ± 59.42 Ab	21.09 ± 2.13 Ac	2.9 ± 0.18 Bc

Note: Different uppercase letters indicate significant differences ($P < 0.05$) between the W and CK treatments at each altitude, and different lowercase letters indicate significant differences ($P < 0.05$) amongst the altitudes in the W or CK treatment. MBC, microbial biomass C; MBN, microbial biomass N; MBP, microbial biomass P. Data are presented as mean ± standard error (n = 3).

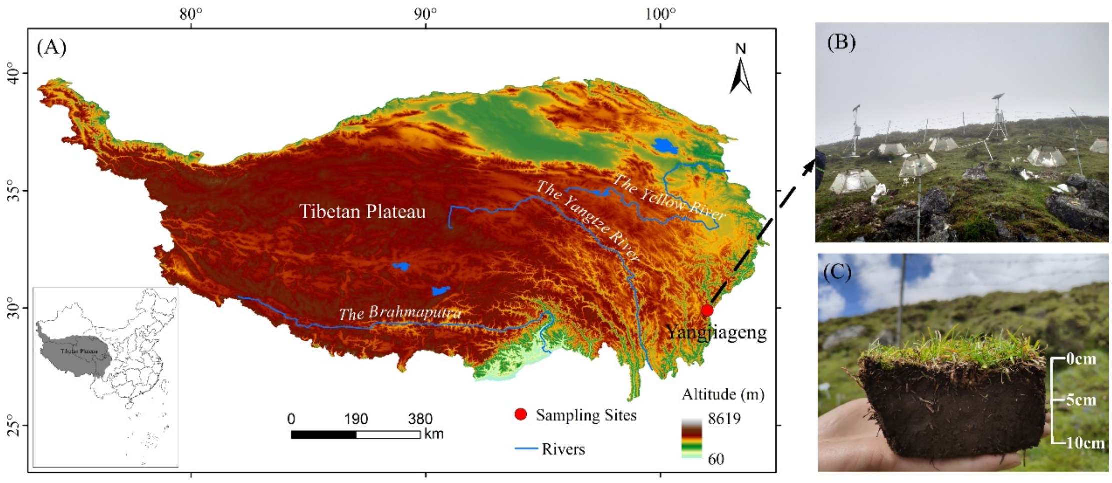

Fig. S1 Study area (A) and photos of the OTC (open-top chamber) warming experiment (B) in an alpine grassland at the eastern edge of the Tibetan Plateau.

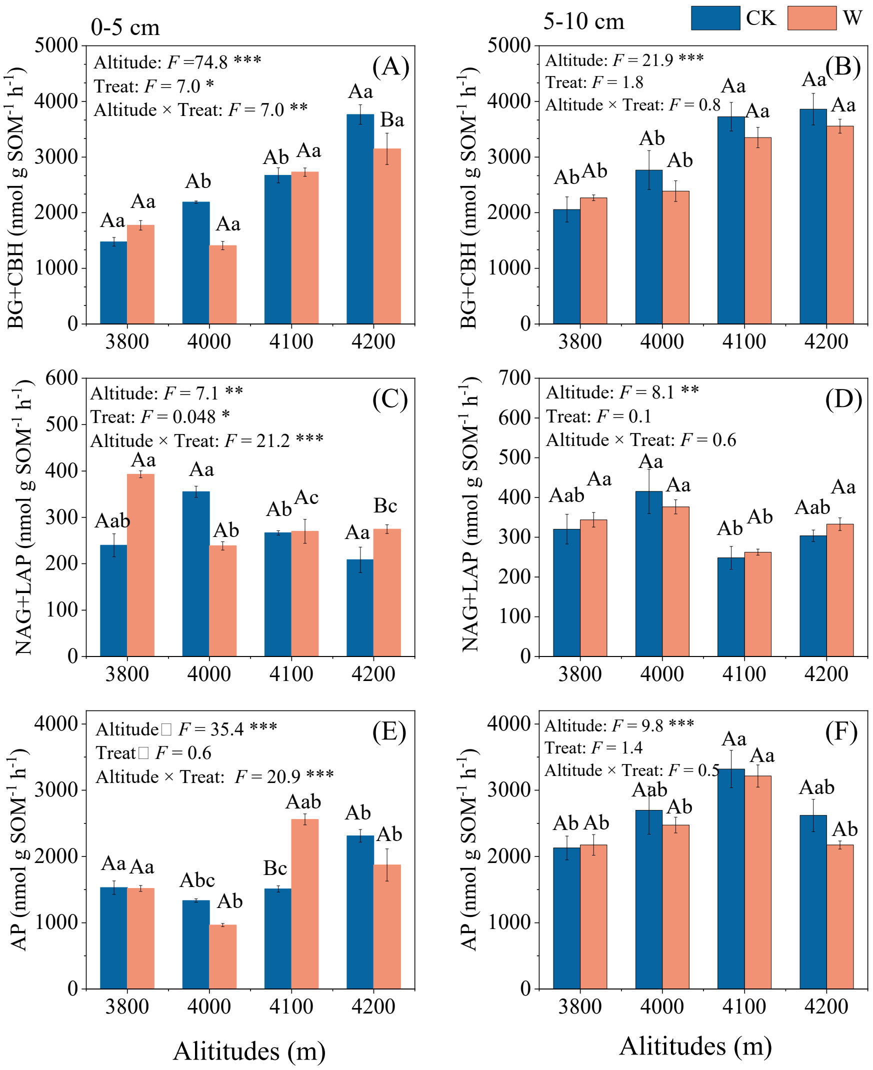

Fig. S2 Extracellular enzyme activity for C-, N- and P-acquiring enzymes in warmed plots (W) and non-warmed plots (CK) along an altitudinal gradient. Error bars indicate standard error of the treatment mean (n=3). Different uppercase letters indicate significant differences ($P < 0.05$) between the W and CK treatments at each altitude, and different lowercase letters indicate significant differences ($P < 0.05$) amongst the altitudes in the W or CK treatment. C-acquiring enzymes, β-1,4-glucosidase (BG) and β-D-cellobiosidase (CBH); N-acquiring enzymes, β-1,4-N-acetylglucosaminidase (NAG) and L-leucine aminopeptidase (LAP); P-acquiring enzyme, acid phosphatase (AP).

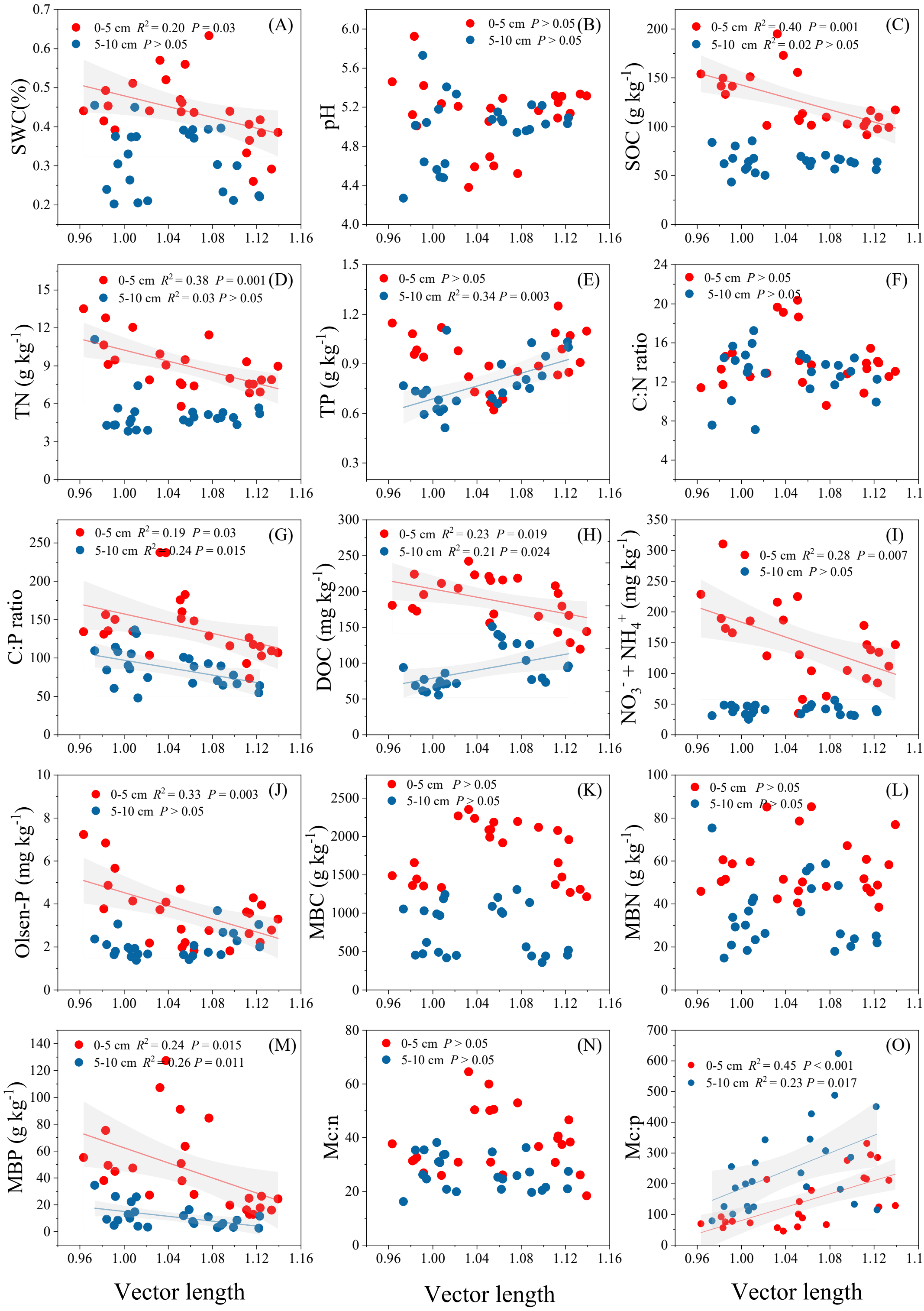

Fig. S3 Vector length in relation to soil physical properties, total nutrient contents, nutrient ratios, available nutrient contents, microbial biomass, and microbial biomass ratios. Solid lines indicate the model fits between the vector lengths and the properties, and grey areas are the 95% confidence intervals of the models.

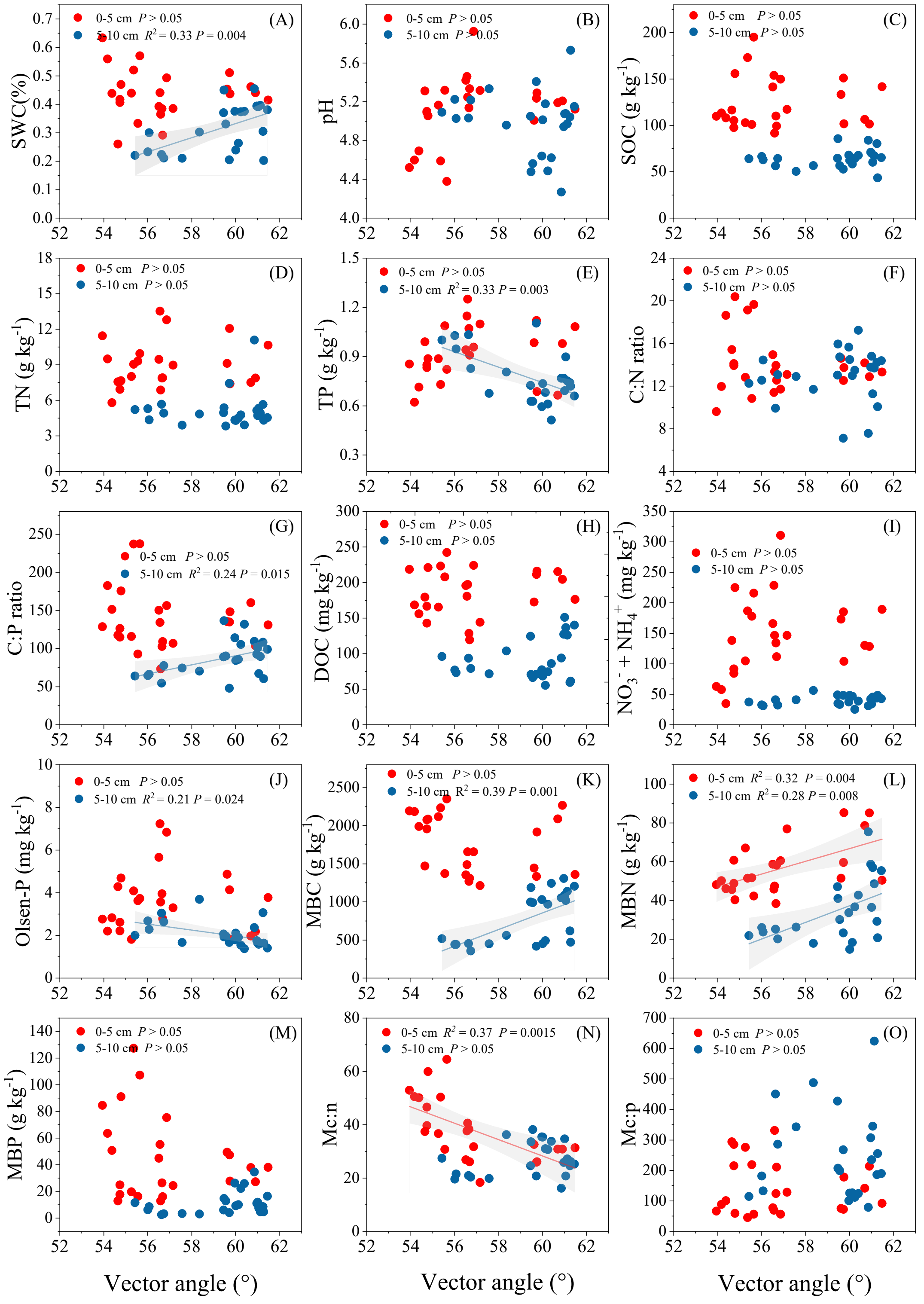

Fig. S4 Vector angle in relation to in relation to soil physical properties, total nutrient contents, nutrient ratios, available nutrient contents, microbial biomass, and microbial biomass ratios. Solid lines indicate the model fits between the vector lengths and the properties, and grey areas are the 95% confidence intervals of the models.