



# Nine years of warming and nitrogen addition in the Tibetan grassland promoted loss of soil organic carbon but did not alter the bulk change in chemical structure

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**Abstract.** Nitrogen (N) and warming effects on ecosystem carbon (C) budgets and stabilization are critical to understand as C sequestration is considered as a mechanism to offset anthropogenic CO<sub>2</sub> emissions, which is important for accurately predicting ecosystem C sequestration and/or potential C loss, remaining controversial though. However, the relevant information, especially for the intervention of environmental controls on grassland soil, is limited in Tibetan Plateau (TP) regions. Here we used a 9-year two-way factorial experiment involving warming with open top chambers (+1.80 °C in the daytime and +0.77 °C in the nighttime at the soil surface) and multilevel nitrogen (N) enrichment treatments (0, 5, 10, and 15 g m<sup>-2</sup> yr<sup>-1</sup>) in the TP to investigate the changes in soil organic carbon (SOC) pool size and chemical structure. Nine-year warming treatment significantly decreased SOC stock in the Tibetan grassland. We observed decreasing SOC concentrations which may be related to changes in the C-degrading enzymes. Surprisingly, the SOC molecular structure remained unchanged in all N-enrichment and warmed plots, suggesting that both treatments had affected all forms of SOC, from simple and complex polymeric in a similar way. Our results suggest that

long-term warming stimulates soil C loss but no preference in SOC loss with different chemical structures.

## 1 Introduction

Soil organic matter is the largest organic carbon reservoir of near-surface terrestrial ecosystems (Dlamini et al., 2016). Even subtle acceleration in soil organic carbon (SOC) decomposition will result in large CO<sub>2</sub> emissions (Davidson and Janssens, 2006). So, knowledge of the factors affecting SOC storage and decomposition is essential for understanding the dynamically changing global C cycle. The influence of global warming on the decomposition of soil carbon has been well documented (Poeplau et al., 2017; Guan et al., 2018; X. Ding et al., 2019), but there remains considerable uncertainty in the potential response of soil C dynamics to the rapid global increase in reactive nitrogen (N, coming largely from agricultural fertilizers and fossil fuel combustion) as well as the combined effects with warming (Liang and Balsler, 2012; Devaraju et al., 2015; Li et al., 2017). For example, soil N availability would strongly affect microbial physiology and C-degrading enzymes (EnC),

which can subsequently alter soil C feedbacks to warming (Mack et al., 2004; Contosta et al., 2015). EnC have been shown to play an important role in SOM nutrient cycling and catabolism (H. Chen et al., 2018), and information on such activity can be used to investigate substrate nutrient demand and responses to environmental changes (Allison et al., 2010; Wang et al., 2015). The knowledge gap demonstrated a need to focus research on biological and physicochemical controls of SOC stabilization and destabilization processes as a basis for understanding causal relationships and key processes that determine pool sizes and turnover rates of functional SOC pools (von Lützow and Kögel-Knabner, 2009).

Soil warming experiments in the field have shown that warming generates a considerable short-term soil C loss (Lu et al., 2013; Romero-Olivares et al., 2017). This loss declines over time (e.g., > 2 years) (Romero-Olivares et al., 2017), although there is evidence that it can continue for longer (e.g., > 20 years) (Melillo et al., 2017). Also, indirect effects of warming on nutrient cycling (Pendall et al., 2004) or plant inputs (Bradford et al., 2016) may have cascading effects on SOC quality and quantity (Lu et al., 2013) and consequently on microbial decomposition of SOC, including recent plant-derived material (Hicks Pries et al., 2017) or older SOC (Vaughn and Torn, 2019). Because ecosystems in alpine meadows are normally N-limited (Hobbie et al., 2002), increased N released from decomposing SOC could stimulate plant productivity, thereby increasing ecosystem C storage (Moscatelli et al., 2008). However, field evidence suggests that soil microbial activity and biomass may also be N-limited in some C-rich ecosystems (Mack et al., 2004; Rinnan et al., 2007). Therefore, increased N released from decomposition of SOC could further fuel microbial activity and decrease soil C storage. In addition, according to the priming effect hypothesis, the increase in N availability and labile C substrates promotes microbial C utilization, thereby increasing the degradation of less decomposable SOC and leading to a negative effect on soil C accumulation over the long term (Riggs and Hobbie, 2016). However, it has proven difficult to quantify bulk SOC stock changes and organic matter composition directly (Sistla et al., 2013; Van Gestel et al., 2018). As alternatives, molecular-level techniques can detect how temperature affects plant and soil organic matter, microbial growth, and their community composition under climate warming (Feng et al., 2008; Xue et al., 2016; Pold et al., 2017).

Since the molecular structure of organic material has long been thought to determine long-term decomposition rates in soil humic substances, solid-state cross-polarization magic-angle spinning carbon-13 nuclear magnetic resonance (CP-MAS  $^{13}\text{C}$  NMR) and diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy have been successfully applied in studies on changes in SOC chemical structure during organic matter decomposition without any physical or chemical destruction (Schmidt et al., 2011). The structure of SOC could be very complex, but by combining both techniques

(solid-state  $^{13}\text{C}$  NMR and DRIFT), complementary information could be obtained on aromatic and aliphatic components (Ferrari et al., 2011).

Despite the importance of the response of SOC stocks to warming and N enrichment in the intact ecosystem, results about the chemical stabilization mechanisms (i.e., molecular structure of SOC) in alpine meadows remained controversial. This knowledge gap is significant because the Tibetan Plateau (TP) stores a large C pool, with 36.6 Pg C stored in the top 3 m of the soil, accounting for 23.5 % of China's total organic soil-stored C and 2.5 % of the global pool of soil C, which is of great importance in regulating future global climate change and C emission (Genxu et al., 2002; J. Ding et al., 2019). At the same time, the TP has experienced climate warming at a rate that is 2 times faster than that in other regions worldwide and is predicted to lead to great soil C losses via microbial respiration in the future (Biskaborn et al., 2019). In addition, during recent decades, the TP has been subject to high levels of N enrichment driven by agricultural activities (up to  $\sim 8.0 \text{ g m}^{-2} \text{ yr}^{-1}$ ) (Gao et al., 2007; Li et al., 2012; Zhang and Fu, 2020) and atmospheric N deposition ( $1 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) (Lü and Tian, 2007; Yu et al., 2019), with an annual rate of increase in deposition ( $0.053 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) (Liu et al., 2013; J. Wang et al., 2019), and this kind of enrichment has been shown to induce soil C loss and affect SOC stabilization in this typical N-constrained ecosystem (Xiao et al., 2021).

Since temperature is one of the main drivers of the vegetation growth and decomposition of organic matter, ongoing climate change may alter biophysical processes, with consequences for ecosystem functioning, especially in highly sensitive cold regions such as the alpine meadow on the TP (Piao et al., 2006; Yang et al., 2008). However, how and to what extent chemical stabilization of SOC shifts may occur, and consequently SOC storage and C-climate feedback would respond to warming and N enrichment in an alpine meadow ecosystem, remain largely unknown. Here, we used soils from a 9-year experiment with a two-way factorial design involving soil warming (daytime:  $1.80^\circ\text{C}$ ; nighttime:  $0.77^\circ\text{C}$ ) and control plots and N enrichments (0, 5, 10, and  $15 \text{ g m}^{-2} \text{ yr}^{-1}$ , marked as N0, N1, N2, and N3, respectively) (Liu et al., 2016) on the TP to examine the changes in the stock and molecular structure of SOC.

We hypothesized that 9-year N enrichment and warming would affect SOC stocks and the chemical structure of the SOC. N enrichment below a certain threshold may favor C sequestration in the alpine grassland ecosystem, but warming may result in C loss. Added N would stimulate hydrolytic enzyme activity, while warming would repress enzyme activity. Finally, we hypothesized that variation in the enzyme response to N and temperature would emerge as an important explanation for variability in the effect of added N and warming on SOC stock.

## 2 Materials and methods

### 2.1 Site description

Plot sampling was conducted in a grassland ecosystem located on the eastern edge of the Tibetan Plateau, Maqu County, Gansu Province, China (35°58' N, 101°53' E; 3500 m above sea level; Fig. 1; NOAA, 2015), in August 2019. The grassland ecosystem of the TP covers an area of about  $1.53 \times 10^6$  km<sup>2</sup>, accounting for nearly 60 % of the total area of the TP (Liu et al., 2016). Alpine meadow is the main vegetation type in this area, and the plant community is dominated by perennial herbaceous species of *Poaceae*, *Ranunculaceae*, and *Asteraceae*. The area of alpine meadow accounts for more than 44 % of the area of alpine grasslands, and its SOC storage accounts for 56 % of the SOC storage of alpine grasslands on the whole TP (Yang et al., 2008). The soil in the alpine meadow is classified as Mat-Cryic Cambisol (Hou et al., 2019). This region has a typical plateau continental climate. The mean annual precipitation is 620 mm, and most falls in the growing season (summer). The mean annual temperature is 1.2 °C, with the lowest monthly mean temperature occurring in January (−10.7 °C) and the highest monthly mean temperature occurring in July (11.7 °C). During the past several decades, the mean annual temperatures in the region have risen at a rate of 0.58 °C per decade (Liu et al., 2016).

### 2.2 Experimental design and soil sampling

A field-based warming experiment was established in June 2011 with a split-plot block design in which both temperature (open-top chamber, +1.80 °C in the daytime and +0.77 °C in the nighttime at the soil surface) and nitrogen (0, 5, 10, and 15 g m<sup>−2</sup> yr<sup>−1</sup>, corresponding to N0, N1, N2, and N3, respectively) were manipulated, with six replicates per treatment (Liu et al., 2016). The 48 plots (8 treatments (N0, N1, N2, N3, WN0, WN1, WN2, WN3) with 6 replicates each treatment) with roughly the same species diversity and community structure were 5 m × 5 m and were separated by 1 m from adjacent edges. Additional details can be found in our previous studies (Sun et al., 2023). Surface layer (0–10 cm) soils were collected from these 48 plots using an auger 4 cm in diameter in August 2019. Then, the fresh soil samples were transported to the laboratory on ice.

### 2.3 Soil analysis

Soil microbial biomass carbon (MBC) was measured according to the chloroform fumigation extraction method using a total organic carbon (TOC) analyzer (Multi N/C 3100, Analytik Jena GmbH, Germany) (Vance et al., 1987). The soil pH was determined in a 1 : 5 soil : water suspension with a pH meter (PHS-3D, Rex, Shanghai, China). Bulk density samples were dried at 105 °C for 48 h and calculated by dividing the oven-dried soil mass by the steel cylinder volume

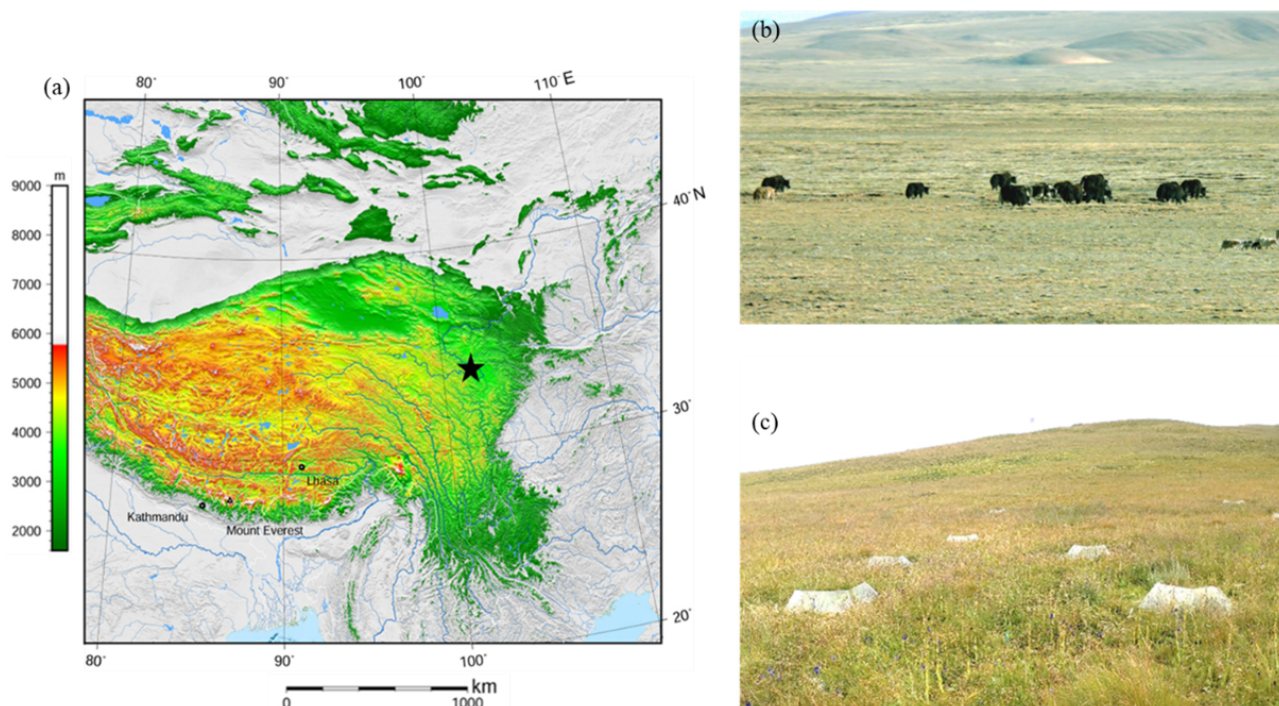
(100 cm<sup>3</sup>) because coarse fragments (stones or large roots) were not obtained in ring samples. For SOC analysis, air-dried soil was ground and HCl-fumigated (Komada et al., 2008), and then the SOC concentration was determined with an elemental analyzer (FlashSmart, Thermo Fisher Scientific, USA). The SOC stocks (0–10 cm) were calculated by multiplying the SOC concentration by the bulk density (Walter et al., 2016). At these 48 sites, all plants in three plots (50 cm × 50 cm) were harvested and dried to determine the aboveground biomass (AGB).

We measured the activity of four extracellular enzymes in the soil at an in situ pH (Nie et al., 2013). The absorbance of the C degradation enzymes β-D-cellubiosidase (CB), α-glucosidase (AG), β-glucosidase (BG), and β-xylosidase (XYL) was measured using a Tecan infinite M200 microplate fluorometer (Grodig, Austria) with 365 nm excitation and 460 nm emission filters. The activities were expressed as dry soil (nmol h<sup>−1</sup> g<sup>−1</sup>). We combined CB, AG, BG, and XYL into an EnC.

### 2.4 SOC molecular structure examination using NMR spectroscopy

The soil samples used for NMR spectroscopy analysis were pre-treated using hydrofluoric (HF) acid (2 %) to eliminate paramagnetic materials, e.g., ferric ions and manganese ions, that may affect the NMR signals (Skjemstad et al., 1994; Schmidt et al., 1997; Mathers et al., 2002). The solid-state NMR spectra (<sup>13</sup>C-CPMAS) were recorded on a Bruker AVANCE III 600 MHz instrument (Bruker Instrument Inc., Billerica, MA, USA). The acquisition conditions were set at a frequency of 75.5 MHz, with 20 kHz spectral width, 5 kHz spinning speed, 2 ms contact time, and 2.5 s recycle time. The regions of 0–210 ppm spectra were plotted.

We examined seven chemical shift regions to represent the main C functional groups (Golchin et al., 1997; Sun et al., 2019). We report proportions of each chemical shift area and calculated four ratios indicative of the characteristics of soil organic matter. The alkyl C, the most persistent fraction of SOC, comes from original plant biopolymers (such as cutin, suberin, and waxes) or from metabolic products of soil microorganisms (Ussiri and Johnson, 2003). As these materials decompose, the relative abundance of O-alkyl C in the litter materials decreases, and there is a progressive increase in alkyl C (Bonanomi et al., 2013). Therefore, the ratio of alkyl C to O-alkyl C ( $A/O-A = C_{0-45}/C_{60-90}$ ) is an index representing the extent of SOC decomposition. The higher this ratio, the higher the decomposition degree of SOC (Wang et al., 2015). Aromaticity ( $C_{110-165}/C_{0-165}$ ) was used to indicate the complexity of a molecular structure (Dai et al., 2001). The ratio of aliphatic C/aromatic C (alip/arom),  $C_{0-110}/C_{110-165}$ , also indicates the molecular structure of soil C, with a higher alip/arom meaning less aromatic nuclear structure in humus. The hydrophobic C/hydrophilic C (HB/HI) ratio,  $(C_{0-45} + C_{110-165})/(C_{45-110} + C_{165-210})$ , was



**Figure 1.** Elevation map of the studied sites (a; the pentagram refers to the sampling point, NOAA, 2015), photo of the alpine meadow (b), and the diagram of the warming treatment (open-top chamber) (c). Note: the map (a) was cited from the Wikimedia Commons website (Tibet and surrounding areas above 1600 m, created using Generic Mapping Tools).

used to reflect the stability of soil aggregation (Spaccini et al., 2006; Wang et al., 2010). The higher values of the HB/HI ratio indicated that SOC was more hydrophobic (Cao et al., 2016), which in turn implied that SOC was more stable (Spaccini et al., 2006; Wu et al., 2014).

## 2.5 Bulk soil organic matter composition using DRIFT spectroscopy

Because of the large number of variables affecting a spectrum, it is extremely difficult to obtain a complete and fine molecular structure from a single spectrum without additional knowledge obtained by other spectroscopic techniques (Ferrari et al., 2011). So, we employed another complementary molecular-level analysis, DRIFT spectroscopy, which is a useful method for the characterization of organic matter (Olk et al., 2000) and humic substances (Mao et al., 2008; Francioso et al., 2009), to explore potential shifts in SOC composition in response to warming and N enrichment. To characterize warming and N-induced changes in SOC composition, 6 mg of a ground soil sample was examined by DRIFT spectroscopy. Mid-infrared spectra were recorded using a Bruker TENSOR 27 spectrometer (Billerica, MA, USA) from 4000 to 400  $\text{cm}^{-1}$  (average of 16 scans per sample at 4  $\text{cm}^{-1}$  resolution). Infrared absorption bands were represented by functional groups. Infrared absorption bands were represented by func-

tional groups as follows: aliphatic C–H (2900  $\text{cm}^{-1}$ ), aromatic esters, carbonyl/carboxyl C=O (1735–1720  $\text{cm}^{-1}$ ), aromatic C=C (1660–1600  $\text{cm}^{-1}$ , 1430–1380  $\text{cm}^{-1}$ ), lignin-like residues (1515–1500  $\text{cm}^{-1}$ ), phenolic/cellulose (1260–1210  $\text{cm}^{-1}$ ), and aromatic C–H (880, 805, and 745  $\text{cm}^{-1}$ ) carbon (Niemeyer et al., 1992; Leifeld, 2006; Chatterjee et al., 2012). A summary of the absorption bands associated with different compound classes can be found in Fig. S2 in the Supplement. Additional details can be found in our previous study (Ofiti et al., 2021).

## 2.6 Statistical analysis

All the data are presented as the mean values of six field replicates. Any significant differences in soil physicochemical properties among the different N enrichment levels and warming treatments were identified by using two-way ANOVA followed by Tukey's heavily significant difference (HSD) post hoc test, with differences considered to be statistically significant at  $P < 0.05$ . The statistical analysis was conducted using SPSS 13.0 and R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria, 2013). To access the direct and indirect effects of external factors on SOC stock, structural equation modeling (SEM) was performed using the R packages *plspm* and *piecewiseSEM* (Li et al., 2020). For this purpose, firstly, all data were tested for normality using the Kolmogorov–Smirnov test, and the

non-normal variables were log-transformed. Secondly, we established a prior model based on prior knowledge of effects and relationships among the driving factors. Finally, we selected the best model based on overall goodness of fits, including the chi-squared ( $\chi^2$ ) statistic, degrees of freedom (dfs), whole-model  $P$  value, goodness-of-fit index, and root-mean-square error of approximation (Schermelleh-Engel et al., 2003).

### 3 Results

#### 3.1 Bulk soil properties

N enrichment and warming showed a significant interaction effect for pH, AGB, EnC, C/N, and SOC stock ( $P < 0.05$ , Fig. 2). AGB, EnC, and SOC stocks increased significantly under N enrichment, but the increment decreased with rising N addition concentration ( $P < 0.05$ , Fig. 2b, c, and f). Warming exacerbates soil acidification and decreased the AGB, EnC, MBC, and SOC stock significantly. Both N enrichment and warming significantly decreased the C/N ratio ( $P < 0.05$ , Fig. 2d). Except for MBC, the N and warming interactions significantly altered soil physicochemical properties (Fig. 2, Table S1 in the Supplement).

#### 3.2 SOC speciation as seen by DRIFT and NMR spectroscopy

Changes in SOC molecular composition became apparent in DRIFT and NMR spectra (Figs. 3 and 4 and Figs. S2 and S3 in the Supplement). In all N-enrichment and warming treatments, there was a statistically nonsignificant change in the SOC composition and molecular structure observed by both DRIFT and NMR spectra. The relative abundance of carbonyl/carboxyl C=O, C=C aromatic compounds, and lignin-like residues decreased slightly after N enrichment, though not significantly. The relative abundance of the phenolic/cellulose groups remained stable in all the individual and interaction treatments (Figs. 3 and S2).

The results of  $^{13}\text{C}$  NMR spectroscopy indicated the relative abundance of different C components (Table 1, Figs. 4 and S3), showing that the proportion of the seven C functional groups did not change in soils under N-enrichment and warming treatments. The relative proportions of the seven C functional groups were stable in the eight treatments in the following abundance order: O-alkyl C (mean 33 %), followed by alkyl C (mean 22 %), aromatic C (mean 12 %), N-alkyl C (11 %), carbonyl C and di-O-alkyl C (mean 8 %), and finally phenolic C (mean 3 %) (Table 1, Fig. S3). The four indexes which can represent the extent of SOC decomposition observed by NMR spectra also showed no significant difference under all the N-enrichment and warming treatments (Fig. 4), suggesting that SOC showed a similar degradation state at all N-level enrichments and warming treatments as well as the interaction effects.

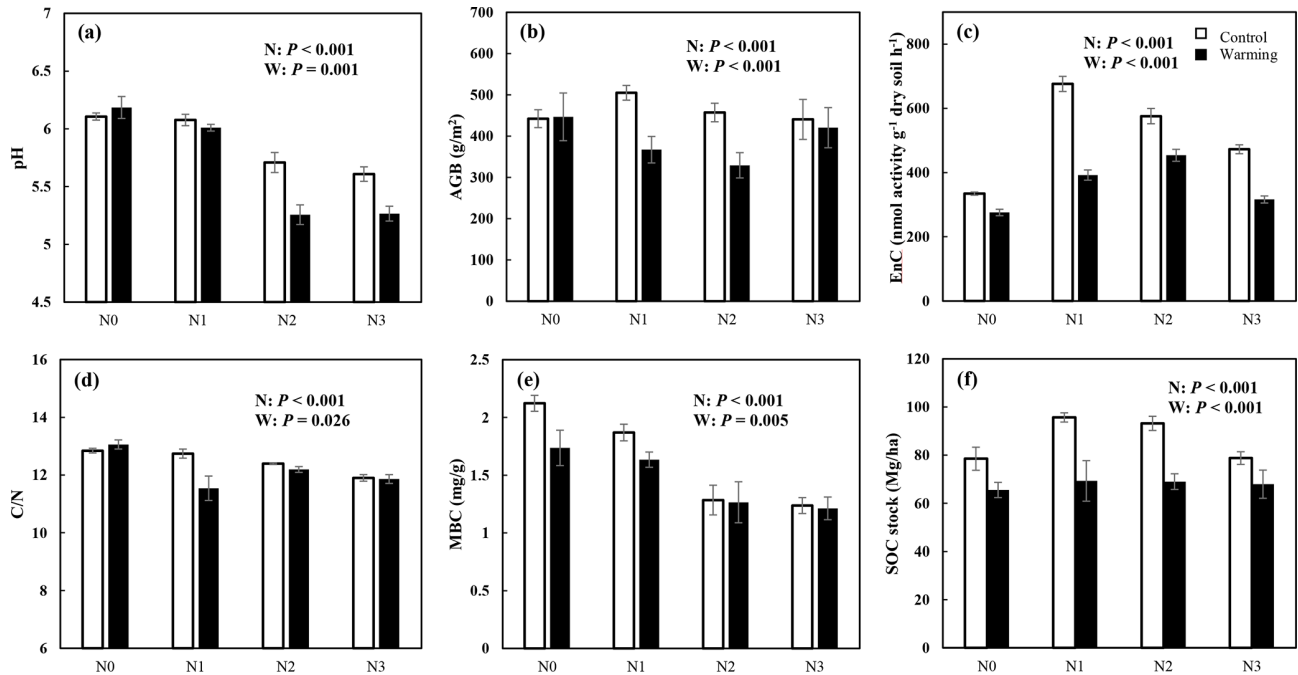
#### 3.3 Factors driving the SOC stocks

We then developed SEM to assess the direct and indirect effects of soil variables on the SOC stocks (Fig. 5). The SEM results revealed strong connections among global change, biotic, and edaphic factors (Fig. 5), demonstrating a need to consider their interactions when predicting SOC stock and its response to individual and interactive effects of N enrichment and warming. Overall, the SEM explained 44 %, 55 %, and 21 % of the variance in SOC stock driven by N enrichment, warming treatment, and interaction effects, respectively. In both N and warming patterns, C-degrading enzymes showed an important indirect factor in regulating SOC stock. N enrichment had a positive effect on SOC stock by enhancing enzyme activities. In contrast, warming had a negative effect on SOC stock by inhibiting microbial enzymes. In addition, warming had a strong negative direct effect on SOC stock (Fig. 5b). However, no significant direct or indirect pathways for the interaction effects of N and warming on SOC stocks were observed (Fig. 5c).

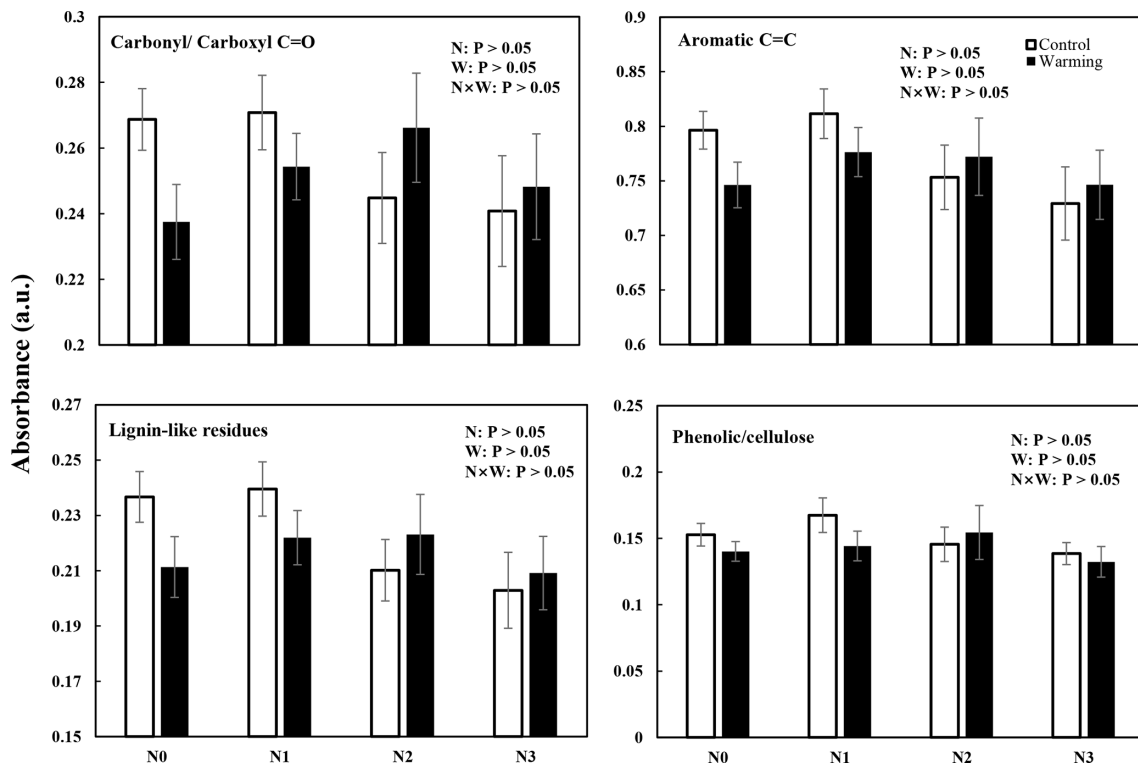
### 4 Discussion

#### 4.1 Effects of warming and N enrichment on soil C pool size

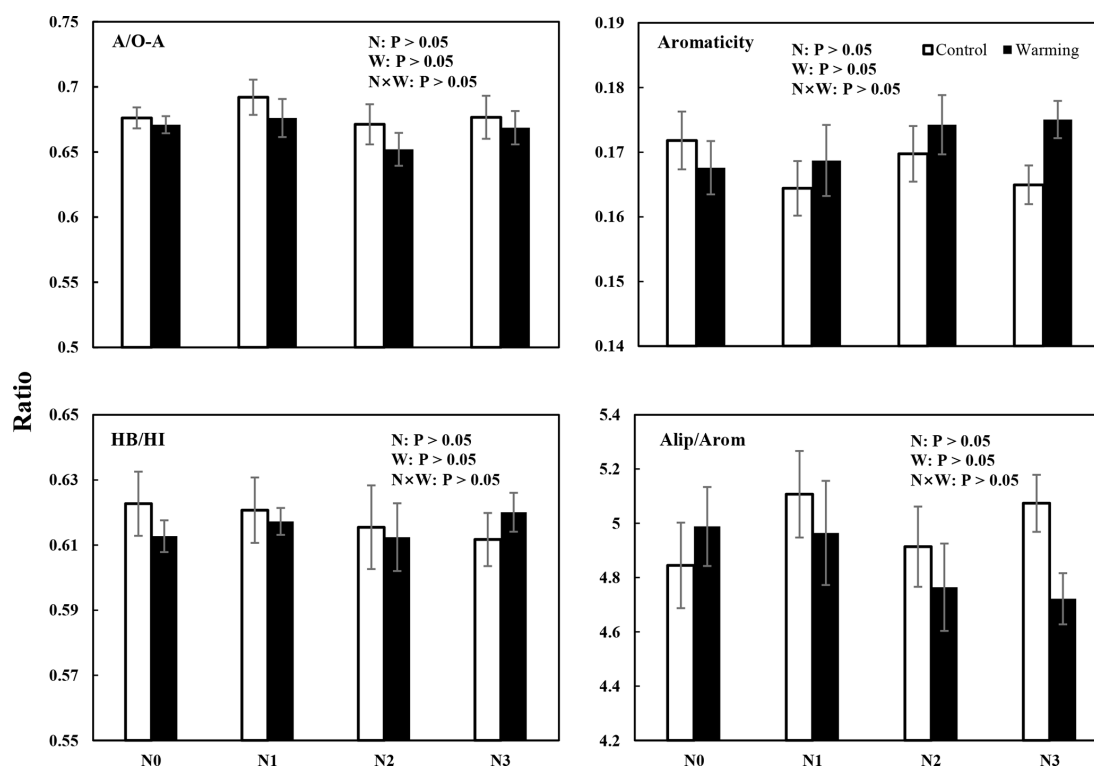
It is suggested that small N inputs can decrease  $\text{CO}_2$  emissions by changing the interaction between plants and soil microbes in N-limited ecosystems, e.g., by increasing plant productivity and root biomass and then organic C inputs to the soil by promoting N availability and thus retarding litter and SOC decomposition (Franklin et al., 2003; Mo et al., 2008; Zhou et al., 2014). However, in an alpine grassland, Jiang et al. (2012) found that both plant growth and microbial activity were generally N-limited, but the ability of plants to capture soil inorganic N was much stronger than that of soil microorganisms. When N was added, increased N availability resulted in increased plant growth, microbial activity, and plant biomass (Micks et al., 2004). Therefore, the decomposition of litter and SOM is enhanced by increasing the quantity of litter input or by elevating microbial activity, and consequently soil functions would shift from C sequestration to C loss. The increased N has consequently reduced the soil pH by 0.26 globally in only 1 decade, which may significantly influence the microbial community composition and activity and then the SOC sequestration capacity (Geisseler and Scow, 2014; Tian and Niu, 2015; Raza et al., 2021). This speculation is consistent with our results that N input below a threshold level (e.g.,  $10 \text{ g m}^{-2}$  in this study) may be beneficial for C sequestration in alpine meadows of the TP and can partly explain the patterns of SOC pool sizes under various N-enrichment levels in this study. Specifically, the SOC stock increased following N enrichment, but as the N addition concentration increased, this growth progressively diminished,



**Figure 2.** N and warming-induced changes in the soil properties (mean  $\pm$  SE,  $n = 6$ ). Control (white bar) and warmed plots (black bar) at four different levels of simulated N deposition. N0, N1, N2, and N3 indicate N enrichments of 0, 5, 10, and 15 g N m<sup>-2</sup> yr<sup>-1</sup>, respectively. The parameters are soil pH (a); AGB, aboveground biomass (b); EnC, C-degrading enzymes (c); C/N, the ratio of soil C concentration to N concentration (d); MBC, microbial biomass carbon (e); and SOC, soil organic carbon stock (f).



**Figure 3.** N and warming-induced changes in the relative abundance of different functional groups identifiable by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy in warmed and control plots (mean  $\pm$  SE,  $n = 6$ ). The spectral regions were assigned to aromatic carbonyl/carboxyl C=O groups, aromatic C=C groups, lignin-like residues, and cellulose/phenolic groups.



**Figure 4.** Four different SOC chemical structural complexity indexes (mean  $\pm$  SE,  $n = 6$ ) from solid-state  $^{13}\text{C}$  CPMAS NMR spectra of soil samples from different treatments. A/O-A = alkyl C/O-alkyl C; HB/HI = hydrophobic C/hydrophilic C; alip/arom = aliphatic C/aromatic C.

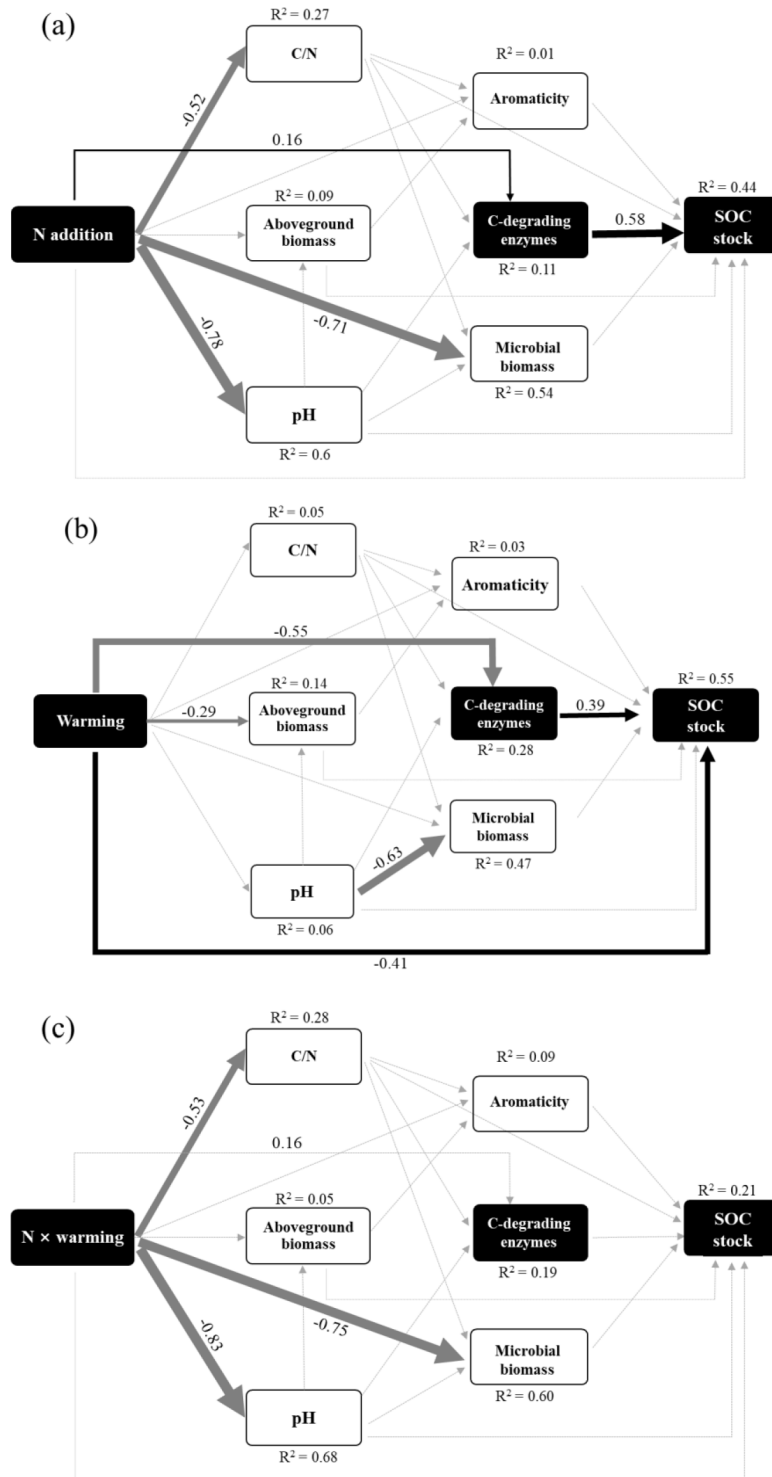
**Table 1.** Relative intensities (mean  $\pm$  SE,  $n = 6$ ) of different carbon chemical shifts from solid-state  $^{13}\text{C}$  CPMAS NMR spectra of soil samples from N and warming treatments.

Chemical shifts		Treatment				
		N0	N1	N2	N3	
$^{13}\text{C}$ NMR (%)	Alkyl C (0–45 ppm)	C	22.64 $\pm$ 0.15	23.22 $\pm$ 0.17	22.55 $\pm$ 0.2	22.85 $\pm$ 0.32
		W	22.68 $\pm$ 0.24	22.75 $\pm$ 0.51	22.07 $\pm$ 0.26	22.29 $\pm$ 0.29
	N-alkyl C (45–60 ppm)	C	10.91 $\pm$ 0.09	11.08 $\pm$ 0.11	10.82 $\pm$ 0.08	10.92 $\pm$ 0.1
		W	10.73 $\pm$ 0.06	10.6 $\pm$ 0.24	10.54 $\pm$ 0.08	10.67 $\pm$ 0.16
	O-alkyl C (60–90 ppm)	C	33.5 $\pm$ 0.35	33.62 $\pm$ 0.53	33.67 $\pm$ 0.54	33.83 $\pm$ 0.47
		W	33.8 $\pm$ 0.3	33.65 $\pm$ 0.23	33.88 $\pm$ 0.45	33.36 $\pm$ 0.31
	Di-O-alkyl C (90–110 ppm)	C	8.74 $\pm$ 0.09	8.65 $\pm$ 0.09	8.9 $\pm$ 0.13	8.82 $\pm$ 0.12
		W	8.88 $\pm$ 0.09	9.02 $\pm$ 0.11	8.88 $\pm$ 0.1	9.02 $\pm$ 0.26
	Aromatic C (110–145 ppm)	C	12.25 $\pm$ 0.33	11.62 $\pm$ 0.26	12.05 $\pm$ 0.3	11.45 $\pm$ 0.28
		W	11.86 $\pm$ 0.36	12.1 $\pm$ 0.45	12.34 $\pm$ 0.38	12.44 $\pm$ 0.21
	Phenolic C (145–165 ppm)	C	3.47 $\pm$ 0.1	3.44 $\pm$ 0.11	3.47 $\pm$ 0.08	3.64 $\pm$ 0.05
		W	3.46 $\pm$ 0.06	3.32 $\pm$ 0.05	3.56 $\pm$ 0.07	3.54 $\pm$ 0.09
	Carbonyl C (165–210 ppm)	C	8.49 $\pm$ 0.1	8.37 $\pm$ 0.17	8.54 $\pm$ 0.18	8.49 $\pm$ 0.3
		W	8.61 $\pm$ 0.16	8.57 $\pm$ 0.24	8.73 $\pm$ 0.14	8.68 $\pm$ 0.19

eventually even disappearing. Our results revealed that alpine grassland ecosystems on the TP may become a potential C source in future scenarios of increasing N enrichment.

In our study presented here, the 9 years of warming resulted in a very significant SOC loss of 14 % to 28 % (Fig. 2

and Fig. S1 in the Supplement). The TP stored large amounts of SOC because of the permafrost soil, where limited C decomposition has led to the accumulation of large SOC stocks (Hengl et al., 2014; Schuur et al., 2015). Previous studies showed that the vulnerability of soils with large C stocks de-



**Figure 5.** The factors regulating the SOC stock under (a) N enrichment, (b) warming treatment, and (c) interactive effects of N and warming. In the SEM analysis, black arrows represent significant positive pathways, gray arrows represent significant negative pathways, and gray dashed arrows indicate nonsignificant pathways. Values next to the arrows represent standardized effect sizes with statistical significance (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ). The thickness of the arrow represents the standardized effect sizes. C-degrading enzyme indicates the sum of  $\beta$ -D-cellubiosidase (CB),  $\alpha$ -glucosidase (AG),  $\beta$ -glucosidase (BG), and  $\beta$ -xylosidase (XYL). Goodness-of-fit statistics for the model are shown as follows: (a)  $\chi^2 = 4.53$ ,  $P = 0.53$ , GFI = 0.99, RMSEA < 0.001; (b)  $\chi^2 = 4.47$ ,  $P = 0.486$ , GFI = 0.99, RMSEA < 0.001.



rives from the high temperature sensitivity of C decomposition and biogeochemical restrictions to the processes driving soil C inputs (Davidson and Janssens, 2006; García-Palacios et al., 2021). In contrast to this, in soils with low initial C stocks, small losses coming from accelerated decomposition induced by rising temperature may be offset by concurrent increases in plant growth and soil C stabilization (Day et al., 2008; Macias-Fauria et al., 2012; Crowther et al., 2015). However, in areas with larger SOC stocks, accelerated decomposition exceeds the potential C accumulation of plant growth, contributing to a significant C loss to the atmosphere (Luo et al., 2019).

#### 4.2 Effects of N enrichment and warming on SOC chemical compositions

SOC chemical composition is controlled not only by the chemistry of the plant material input into the soil, but also by the microbial processing and degradation of SOC (Baldock et al., 1992). Although N addition can stimulate plant growth and increase litter fall, it can also accelerate or slow down microbial processing of plant residues, thus altering the chemical composition of SOC (J.-J. Wang et al., 2019). Surprisingly, we observed that the SOC molecular structure remained unchanged in all N-enrichment and warmed plots (Figs. 3 and 4).

As the predominant chemical component of SOC across all treatments in our study, O-alkyl C is mainly composed of carbohydrates, peptides, and other labile organic components derived from the fresh material, which could be preferentially degraded compared with more resistant components such as alkyl C (Simpson and Simpson, 2012; He et al., 2018). So, we hypothesized that this result could indicate that N and warming may have the same impact on the input of fresh plants on the TP. Unlike O-alkyl C, lipids represent the main source of alkyl C (aliphatic chains), which is derived from the original plant biopolymers. Lignin and tannin represent the main source of aromatic C, together with phenolic C, mainly originating from lignin and amino acids of peptides (Baldock et al., 1992). All these components are more resistant to microbial decomposition than labile O-alkyl C (Simpson and Simpson, 2012). Our results suggested that the proportions of the stable SOC chemical structures remained the same between the different N-enrichment concentrations and warming treatments, indicating the synchronous degradation of SOC. The alkyl/O-alkyl ratio and aromaticity, normally regarded as the indicator of the relative stage of SOC degradation and widely used as an indicator to reflect the complexity of SOC chemical structures (Baldock et al., 1992), exhibited no significant difference among N-enrichment or warming treatments, suggesting that all N levels and warming treatments exerted similar effects on the degradation of SOC and aromatic and complex molecular structures (Zhang et al., 2013).

Infrared spectroscopy of SOC showed a statistically non-significant change in the treated plots and the control plots, consistent with a previous study which showed that the 4.5 years of +4 °C whole-soil warming did not change the relative abundance of carbonyl/carboxyl C=O and C=C aromatic compounds in the surface soils (above 20 cm) from a forest (Ofiti et al., 2021). Collectively, the above results suggested that the molecular structure of surface SOC may not be as sensitive to long-term warming as we thought before (Atanassova and Doerr, 2011; H. Chen et al., 2018). Surface SOC is dominated by recent (less transformed) plant-litter inputs, which is less degraded and transformed than sub-soil SOC (Ofiti et al., 2021). The lack of change in plant- and microorganism-derived organic matter in the surface soil may be due to slight drying and warming near the surface (Soong et al., 2021), which could have inhibited or resulted in relatively fewer surface inputs. We noticed that warming significantly reduced AGB under N1 and N2 enrichments in this study (Fig. 2). Compared with labile SOC, stable SOC can be more vulnerable to priming once microbes are provided with exogenous C substrates. This high vulnerability of stable SOC to priming warrants more attention in future studies on SOC cycling and global change (Zhang et al., 2022). Overall, the stabilized functional SOC molecular structure suggests that soil warming and N enrichment had similarly affected the labile and stabilized SOC of this C-rich grassland soil at the level of chemical stability of organic C molecules, along with the C loss.

#### 4.3 Regulating factors of SOC stock

Our interpretation that prolonged warming could reduce SOC storage is further supported by the simultaneous reduction in different C pool sizes characterized by various chemical structural complexities with long-term warming. Many previous studies have shown that microorganisms preferentially use the labile C pool for community utilization and turnover after short-term warming (Melillo et al., 2002; Kirschbaum, 2004). However, after the initial microbial assimilation of readily accessible SOC with warming, soil microorganisms can acclimate to C starvation through utilization of chemically less available C with continued warming (Chen et al., 2020). This transformation in microbial preferences of C substrates can be facilitated by changes in C-degrading enzyme activities (Crowther and Bradford, 2013).

Our results indicated that C-degrading enzymes could play a key role in regulating soil C storage (Fig. 5a and b), which is in line with previous explanations for continued soil C loss with long-term warming, such as shifts in microbial community and physiology (Melillo et al., 2017; Metcalfe 2017), changes in microbial carbon use efficiency (Tucker et al., 2013), and increased microbial accessibility to litter and SOC (Doetterl et al., 2015; Bailey et al., 2019), which are all closely related to changes in microbial C-degrading enzyme activities. For example, warming decreased the abundance

of lignin-derived compounds but increased ligninase activity in a mixed temperate forest (Feng et al., 2008). Although only cellulase activity was measured in our study, a previous meta-analysis study has shown significantly increasing ligninase activity after warming, enhancing the evidence of microbial response to recalcitrant C pools and the evidence of simultaneous loss of different C fractions after long-term warming (J. Chen et al., 2018). Microbial utilization of recalcitrant C pools could substantially accelerate overall soil C loss. This is because depolymerization of these recalcitrant macromolecules increases microbial accessibility to litter and SOC that was protected by recalcitrant C pools before (Schmidt et al., 2011; Lehmann and Kleber, 2015; Paustian et al., 2016).

While N fertilization exerts both direct and indirect impacts on SOC, its influence on carbonates is direct, leading to continuous losses. This not only serves as a source of atmospheric CO<sub>2</sub> (Kim et al., 2020; Raza et al., 2020; Zamanian et al., 2018) but also degrades soil structure and affects physical, chemical, and biological properties (Meng and Li, 2019). Under acidic conditions, this process induces fundamental changes in microbial community composition and enzyme activity critical for SOC stability (Rowley et al., 2020). In ecosystems characterized by N limitation, such as alpine grasslands, N enrichment enhances N availability, accelerating the decomposition of labile organic C, especially in these otherwise C-rich ecosystems. This in turn results in decreased soil C availability (Craine et al., 2007; Janssens et al., 2010; Song et al., 2017). A previous study at our research site revealed a significant reduction in the soil labile C pool within the particulate organic C fraction with increasing N enrichment, signifying a decline in soil C availability (Chen et al., 2019). Our findings demonstrate that N enrichment significantly stimulates EnC activities and enhances microbial demand for C (Fig. 2), aligning with prior research indicating that added N stimulates the activity of soil cellulose-degrading enzymes (e.g., CB and BG) (Carreiro et al., 2000; Saiya-Cork et al., 2002; Chen et al., 2017). This stimulation may be attributed to the increase in C-acquiring enzymes resulting from heightened microbial demand for C, especially in N-limited ecosystems (Keeler et al., 2009). Previous studies suggest that N enrichment could induce C limitation by reducing plant allocation to fine root production, leading to lower C input into the soil (Treseder, 2008). Thus, we propose that factors beyond the thermal environment, such as N enrichment, can modulate soil enzymes and alter substrate availability. Moreover, these processes can mediate the strength of the soil C–climate feedback. Although N enrichment may increase soil C sinks, this increase may be counteracted by warming. In summary, our results suggest that warming and N enrichment have antagonistic interaction effects on SOC stock, with differential effects on the contribution of alpine meadows to the soil C pool, which may explain the result that the N and warming interaction did not show significant direct or indirect effects on SOC storage in

the SEM results. All these results underscore the importance of considering soil C availability and enzymatic activity responses, which collectively determine the response of the C balance to multiple environmental changes, for a more comprehensive understanding of C storage dynamics.

## 5 Conclusion

Based on a 9-year warming (+1.80 °C in the daytime and +0.77 °C in the nighttime at the soil surface) and different levels of N-enrichment experiments (0, 5, 10, and 15 g m<sup>-2</sup> yr<sup>-1</sup>), we examined the responses of SOC stocks and their molecular components in a Tibetan alpine meadow ecosystem. In summary, our results show little effects of soil warming and N enrichment on the chemical composition of bulk soil despite ongoing C loss in the warmed plots of the study site (Fig. 2). The SOC molecular structure suggested that the easily decomposable and stabilized SOC is synchronously affected after 9-year warming and N treatments despite the large changes in SOC stocks. Given the long residence time of some SOC (Schmidt et al., 2011), the similar loss of all measurable chemical forms of SOC under global change treatments could have important climate consequences. While we found little effects of soil warming on SOC chemistry and the molecular structure of bulk soil, consistent and long-lasting changes could appear with prolonged soil warming and decreasing SOC stocks in the following years. In this process, the importance of enzyme activity must be emphasized, which has been found to be an important indirect factor in regulating changes in SOC stocks.

*Data availability.* The data that support the findings of this study and those not presented within the article and its Supplement are available from <https://doi.org/10.5281/zenodo.8289311> (Sun et al., 2023b).

*Supplement.* The supplement related to this article is available online at: <https://doi.org/10.5194/bg-21-575-2024-supplement>.

*Author contributions.* MN developed the original ideas presented in the paper. HS performed the overall analysis with assistance from JintL, JinqL, and NOEO. XL and SZ organized the field experiment. HS, MWIS, and MN wrote the first draft, and all the authors jointly revised the manuscript.

*Competing interests.* The contact author has declared that none of the authors has any competing interests.

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