Nine years of warming and nitrogen addition in the Tibetan grassland promoted loss of soil organic carbon but did not alter the bulk change of 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_2 emissions, which is important for accurately predicting ecosystem C sequestration and/or potential C loss, remaining controversial though. ~~Understanding the changes in soil organic carbon (SOC) storage and chemical stabilization dynamics is important for accurately predicting ecosystem C sequestration~~

23 ~~and/or potential C lo~~B~~ss, b~~ut the relevant information, especially for the intervention of

24 environmental controls on grassland soil is limited in Tibetan plateau (TP) regions.

25 Here we used a 9-year two-way factorial experiment involving warming with open top

26 chambers (+1.80 °C in the daytime and +0.77 °C in the nighttime at the soil surface)

27 and multilevel nitrogen (N) enrichment treatments (0, 5, 10, and 15 g m^{-2} year^{-1}) in the

28 TP ~~Tibetan plateau~~ to investigate the changes in SOC pool size and chemical structure.

29 9-year warming treatment significantly decreased SOC stock in the Tibetan grassland.

30 We observed decreasing SOC concentrations which may be related to changes in the C

31 degrading enzymes. Surprisingly, the SOC molecular structure remained unchanged in

32 all N enrichment and warmed plots, suggesting that both treatments had affected all

33 forms of SOC, from simple and complex polymeric in a similar way. Our results suggest

34 that long-term warming stimulates soil C loss but no preference in SOC loss with

35 different chemical structure.

36 **Keywords:** global warming, nitrogen deposition, SOC, molecular structure, C

37 stabilization

1. Introduction

Soil organic matter is the largest organic carbon reservoir of near-surface terrestrial ecosystem (Dlamini et al. 2016). Even subtle acceleration in SOC decomposition will result in large CO_2 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 decomposition of soil carbon has been well documented (Poeplau et al. 2017, Guan et al. 2018, Ding et al. 2019b), 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 Balser 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 & Cooper 2015). EnC has been shown to play an important role in SOM nutrient cycling and catabolism (Chen et al. 2018a), and information on such activity can be used to investigate substrate nutrient demand and response 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 meadow 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. Besides, 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 been proven

difficult to quantify bulk SOC stocks 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 CPMAS ^{13}C NMR

~~spectroscopy~~ and diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy

has been successfully applied in studies on changes of SOC chemical structure during

organic matter decomposition without any physical or chemical destruction (Schmidt

et al. 2011). ~~However, 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~~

~~called diffuse reflectance infrared Fourier transform (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.~~ The structure of SOC could be

very complex but by combining both techniques (~~DRIFT and~~ solid-state ^{13}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.~~Despite the~~

~~importance of the response of SOC stocks to warming and N enrichment in the intact~~

ecosystem, this has not been assessed empirically in alpine meadows. 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, Ding et al.

2019a). At the same time, the TP Tibetan Plateau has experienced climate warming at

a rate that is two 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 Tibetan Plateau has been subject to high

levels of N enrichment driven by agricultural activities (up to ~8.0 g m^{-2} y^{-1}) (Gao et al.

2007, Bo et al. 2012, Zhang and Fu 2020) and atmospheric N deposition (1 g N m^{-2} y^{-1}) (Lü and Tian 2007, Yu et al. 2019) with an annual rate of increase in deposition (0.053

g N m^{-2} y^{-1}) (Liu et al. 2013, Wang et al. 2019b), 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, on-going 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 Tibetan plateau (Piao et al. 2006,

Yang et al. 2008). However, how and to what extent physical 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, remains largely

125   unknown. Here, we used soils from a 9-year experiment with a two-way factorial design

126   involving soil warming (daytime: 1.80°C; nighttime: 0.77°C) and control plots and N

127   enrichments (0, 5, 10, and 15 g m^{-2} y^{-1}; marked as N0, N1, N2, and N3, respectively)

128   (Liu et al. 2016) on the TP ~~Tibetan Plateau~~ to examine the changes in the stock and

129   molecular structure of SOC.

130   We hypothesized that 9-years N enrichment and warming would affect SOC stock and

131   the chemical structure of the SOC. N enrichment below a certain threshold may favor

132   C sequestration in the alpine grassland ecosystem but warming may result in the C loss.

133   And added N would stimulate hydrolytic enzyme activity while warming would repress

134   enzyme activity. Finally, we hypothesized that variation in enzyme response to N and

135   temperature would emerge as an important explanation for variability in the effect of

136   added N and warming on SOC stock.

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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 (101°53′ E, 35°58′ N, 3500 m above sea level, Figure 1 (NOAA 2015)), in August 2019. The grassland ecosystem of the TP covers an area of about 1.53 million km^2, 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, 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 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). ~~The plant community is dominated by perennial herbaceous species of Poaceae, Ranunculaceae, and Asteraceae.~~

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

161   and +0.77°C in the nighttime at the soil surface) and nitrogen (0, 5, 10, and 15 g m^{-2} y^{-}

162   ^{1}, corresponding to N0, N1, N2, and N3, respectively) were manipulated, with six

163   replicates per treatment (Liu et al., 2016). The 48 plots (8 treatments (N0, N1, N2, N3,

164   WN0, WN1, WN2, WN3) with 6 replicates each treatment) with roughly the same

165   species diversity and community structure were 5 × 5 m and were separated by 1 m

166   from adjacent edges. Additional details can be found in our previous studies (Sun et al.,

167   2023). Surface layer (0-10 cm) soils were collected from these 48 plots using a 4-cm-

168   diameter auger in August 2019. Then, the fresh soil samples were transported to the

169   laboratory on ice.

170   **2.3 Soil analysis**

171   Soil microbial biomass carbon (MBC) was measured according to the chloroform

172   fumigation extraction method using a TOC analyzer (Multi N/C 3100, Analytik Jena

173   GmbH, Germany) (Vance et al. 1987). The soil pH was determined in a 1:5 soil: water

174   suspension with a pH meter (PHS-3D, Rex, Shanghai, China). Bulk density samples

175   were dried at 105 °C for 48 h and calculated by dividing the oven-dried soil mass by

176   the steel cylinder volume (100 cm^{3}) because coarse fragments (stones or large roots)

177   were not obtained in ring samples. For soil organic carbon (SOC) analysis, air-dried

178   soil was ground and HCl-fumigated (Komada et al. 2008), and then the SOC

179   concentration was determined with an elemental analyzer (FlashSmart, Thermo Fisher

180   Scientific, USA). The SOC stocks (0-10 cm) were are calculated by multiplying the

181   SOC concentration by the bulk density (Walter et al. 2016). At these 48each sites, all

plants in three plots (50 × 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) were measured using a Tecan infinite M200 microplate fluorometer (Grodig, Austria) with 365 nm excitation and 460 nm emission filters. The activities were expressed in units of nmol $h^{-1} g^{-1}$ dry soil. We combined CB, AG, BG and XYL into a C-degrading enzyme variable (EnC).

2.4 5 SOC molecular structure examination using NMR spectroscopy

The soil samples used for NMR spectroscopy analysis were pretreated using HF (2%) to eliminate paramagnetic materials, e.g. ferric ion and manganese ion, that may affect the NMR signals (Skjemstad et al. 1994, Schmidt et al. 1997, Mathers et al. 2002). The solid-state NMR spectra (^{13}C-CP-MAS) were recorded on a Bruker AVANCE III 600 MHz instrument (Bruker Instrument Inc., Billerica, MA, USA). The acquisition conditions were set at frequency of 75.5 MHz, with 20 kHz spectra 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 4 ratios indicative for 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 represents 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 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 higher Alip/Arom means 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 used to reflect the stability of soil aggregation (Spaccini et al. 2006, Wang et al. 2010). The higher values of 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~~4 Bulk soil organic matter composition using DRIFT spectroscopy

~~However, b~~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 called diffuse reflectance infrared Fourier transform (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/N-induced changes in SOC composition, 6 mg of ground soil sample was examined by diffuse reflectance infrared Fourier transform spectroscopy (DRIFT). Mid-infrared spectra were recorded using a Bruker TENSOR 27 spectrometer (Billerica, Massachusetts, USA) from 4000 to 400 cm^{-1} (average of 16 scans per sample at 4 cm^{-1} resolution). Infrared absorption bands were represented by functional groups.

Infrared absorption bands were represented by functional groups as follows: aliphatic C–H (2900 cm^{-1}), aromatic esters, carbonyl/carboxyl C– –O (1735–1720 cm^{-1}), aromatic C– –C (1660–1600 cm^{-1}, 1430–1380 cm^{-1}), lignin-like residues (1515–1500 cm^{-1}), phenolic/cellulose (1260–1210 cm^{-1}), and aromatic C–H (880, 805, 745 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 Figure S2.

Additional details can be found in our previous studies (Ofiti et al. 2021).

2.5 SOC molecular structure examination using NMR spectroscopy

The soil samples used for NMR spectroscopy analysis were pretreated using HF (2%) to eliminate paramagnetic materials, e.g. ferric ion and manganese ion, that may affect the NMR signals (Skjemstad et al. 1994, Schmidt et al. 1997, Mathers et al. 2002). The solid-state NMR spectra (^{13}C-CP-MAS) were recorded on a Bruker AVANCE III 600 MHz instrument (Bruker Instrument Inc., Billerica, MA, USA). The acquisition conditions were set at frequency of 75.5 MHz, with 20 kHz spectra 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 4 ratios indicative for 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 represents 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 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 higher Alip/Arom means 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 used to reflect the stability of soil aggregation (Spaccini et al. 2006, Wang et al. 2010).

2.6 Regulating factors of SOC indicated by structural equation model

To access the direct and indirect effects of external factors on SOC stock, structural equation modeling (SEM) was performed using the R package 'plspm' and 'piecewiseSEM' (Li et al. 2020). For this purpose, firstly, all data were tested for

2.6~~7~~ Statistical analysis

All 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 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 package '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-square (χ^2) statistic, degrees of freedom (df), whole-model *P* value, goodness of fit index, and the root-mean-square error of approximation (Schermelleh-Engel et al. 2003).

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3. Results

3.1 Bulk soil properties

N enrichment and warming have significant interaction on pH, AGB, EnC, C/N and SOC stock ($P < 0.05$, Figure 2). ~~Soil bulk density, SOC concentration, SOC stock, AGB,~~ ~~and~~ EnC and SOC stock increased significantly under N enrichment but the increment decreased with rising N addition concentration ($P < 0.05$, Figure 2b, 2c, 2f). ~~decreased with N enrichment level as well as warming treatment ($P < 0.05$, Figure 2, Table S1, Figure S1).~~ Warming exacerbates soil acidification and decreased the AGB, EnC, MBC and the SOC stock significantly. ~~Effects of N enrichment and warming treatment on soil properties were shown in the Figure 2 and Table S1. Warming aggravated N-induced soil acidification and microbial biomass C loss ($P < 0.05$, Figure 2a, 2e, 2f). Soil bulk density, SOC concentration, SOC stock, AGB and EnC increased significantly under N enrichment but decreased with N enrichment level as well as warming treatment ($P < 0.05$, Figure 2, Table S1, Figure S1).~~ Both N enrichment and warming significantly decreased C/N ratio ($P < 0.05$, Figure 2d). Except for MBC, the NW interactions significantly altered soil physicochemical properties (Figure 2, Table S1).

3.2 SOC speciation as seen by DRIFT and NMR spectroscopy

Changes in SOC molecular composition became apparent in diffuse reflectance infrared Fourier transform (DRIFT) and nuclear magnetic resonance (NMR) spectra (Figure 3, 4 and Figure S2, S3). In all N enrichment and warming treatments, there was a statistically non-significant change in the SOC composition and molecular structure observed by both DRIFT and NMR spectra. The relative abundance of

315 carbonyl/carboxyl C=O, ~~and~~ C=C aromatics compounds as well as lignin-like residues

316 decreased slightly after N enrichment, not significantly though. The relative abundance

317 of the phenolic/cellulose remained stable in all individual and interaction treatments

318 ~~decreased (non-significant) after N enrichment, however, kept steady in warming plots~~

319 (Figure 3 and S2).

320 The results of ^{13}C NMR spectroscopy indicated the relative abundance of different C

321 components (Table 1, Figure 4 and S3), showing that the proportion of the seven C

322 functional groups did not change in soils under N enrichment and warming treatments.

323 The relative proportions of the seven C functional groups were ~~similar~~stable in the 8

324 treatments in the following abundance order: O-alkyl C (mean 33%), followed by alkyl

325 C (mean 22%), aromatic C (mean 12%), N-alkyl C (11%), carbonyl C and di-O-alkyl

326 C (mean 8%), and finally phenolic C (mean 3%) (Table 1, Figure S3). The four indexes

327 which can represent the extent of SOC decomposition observed by NMR spectra also

328 showed no significant difference under all the N-enrichment and warming treatments

329 (Figure 4), suggesting that SOC showed a similar degradation state at all N level

330 enrichments and warming treatments as well as the interaction effects.

331 **3.3 Factors driving the SOC stocks**

332 We then developed a structural equation model (SEM) to assess the direct and

333 indirect effects of soil variables on the SOC stocks (Figure 5). The SEM results revealed

334 strong connections among global change, biotic, and edaphic factors (Figure 5),

335 demonstrating a need to consider their interactions when predicting SOC stock and its

336 response to individual and interactive effects of N enrichment and warming~~N~~

337 ~~enrichment and warming~~. Overall, the SEM explained 44%, ~~and~~ 55% and 21% of the

338 variance in SOC stock driven by N enrichment ~~and~~, warming treatment and the

339 interaction effects, respectively. In both N and warming patterns, C-degrading enzymes

340 showed an important indirect factor in regulating SOC stock. N enrichment had a

341 positive effect on SOC stock by enhancing enzyme activities. In contrast, warming had

342 a negative effect on SOC stock by inhibiting microbial enzymes. Besides, warming had

343 a strong negative direct effect on SOC stock (Figure 5b). However, no significant direct

344 or indirect pathways for the interaction effects of N and warming on SOC stock were

345 observed (Figure 5C).

346

4. Discussion

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

It is suggested that small N inputs can decrease CO_2 emissions by changing the interaction between plants and soil microbes in N-limited ecosystems, for example, by increasing plant productivity and root biomass and then organic C inputs to the soil by promoting N availability and thus retard 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 one decade, which may significantly influence the microbial community composition and activity and then 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 ~~the critical level~~a threshold level (for example, 10 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 size 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, eventually

even disappearing. Our results revealed that alpine grassland ecosystems on the TP may become a potential C source under 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 % (Figure 2 and S1). The TP ~~Tibetan plateau~~ 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 derives from the high temperature sensitivity of C decomposition and biogeochemical restrictions on the processes driving soil C inputs (Davidson and Janssens 2006; García-Palacios et al. 2021). Contrast with that, 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 not only is controlled by the chemistry of the plant materials input to 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 (Wang et al. 2019a). Surprisingly we

observed that the SOC molecular structure remained unchanged in all N enrichment

and warmed plots (Figure 3, 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~~Tibetan plateau~~. Unlike O-alkyl C, lipids represent the main source of alkyl C (aliphatic chains), which is derived from 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 has been widely used as an indicator to reflect the complexity of SOC chemical structure (Baldock et al. 1992), exhibited no significant difference among N enrichment or warming treatments~~no change after 9-year N enrichment and warming~~, suggesting that all N levels and warming treatments exerted similar effects on the degradation of SOC and aromatic and complex molecular structure (Zhang et al. 2013).

Infrared spectroscopy of SOC showed a statistically non-significant change in the treated plots and the control plots, which were consistent with a previous study that showed the 4.5 years of +4 °C whole-soil warming did not change the relative abundance of carbonyl/carboxyl C=O, and C=C aromatics compounds in the surface soils (above 20 cm) from a forest (Ofiti et al. 2021). Collectively, the above results suggested that molecular structure of surface SOC may not be as sensitive to long-term warming as we thought before (Atanassova and Doerr 2011, Chen et al. 2018a). Surface SOC is dominated by recent (less transformed) plant-litter inputs, which is less degraded and transformed than subsoil 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 less surface inputs. We noticed that warming significantly reduced ~~aboveground biomass~~AGB under N1 and N2 enrichments in this study (Figure 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, ~~stable functional SOC molecular structure indicated that soil warming and N enrichment had similarly affected easily decomposable and stabilized SOC of this C-rich grassland soil despite the C loss.~~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 of different C pool sizes characterized with various chemical structural complexity 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 chemical less available C with continued warming (Chen et al. 2020). This transformation in microbial preference of C substrates can be facilitated by changes in C-degrading enzyme activities (Crowther and Bradford 2013).

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Our results indicated that C-degrading enzymes could play a key role in regulating soil C storage (Figure 5a, 5b), 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 (Chen et al. 2018b). 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 previously 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_2 (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 permafrost and peatland regions, 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 extracellular enzyme (EnC) activities and enhances microbial demand for C (Figure 2), aligning with

prior research indicating that added N stimulates the activity of soil cellulose-degrading enzymes (e.g., cellobiosidase (CB) and β-glucosidase (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 NW 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.

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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 level N enrichment experiment (0, 5, 10, and 15 g m^{-2} year^{-1}), 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 (Figure 2). The SOC molecular structure suggested that the easily decomposable and stabilized SOC are synchronously~~similarly~~ 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. ~~Permafrost soils contain half of global SOC stocks (Ding et al. 2016, Hugelius et al. 2020).~~ While we found little effects of soil warming on SOC chemistry and 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 Supplementary Information file are available from https://doi.org/10.5281/zenodo.8289311.

Author contributions

M.N. developed the original ideas presented in the manuscript; H.S. performed the overall analysis with the assistance from Jintao Li. and Jinquan Li, N. O.; X.L. and S.Z. organized the field experiment; H.S., M.S. and M.N. wrote the first draft, and all 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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Figure and table legends

Figure 1. Elevation map~~Location~~ of the studied sites (a, the pentagram refers to the sampling point)~~.~~, photo of the alpine meadow (b) and the ~~–~~diagram of the warming treatment (open-top chamber) (c).

Figure 2. N and warming-induced changes in the soil properties (mean ± 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^{-2} year^{-1}, respectively. Parameters are: Soil pH (a); AGB, aboveground biomass (b); EnC, C-degrading enzymes (c); C/N, ratio of soil C concentration to N concentration (d); MBC, microbial biomass carbo (e); SOC, soil organic carbon stock (f).

Figure ~~3~~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 ± 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. ~~No significant differences were found.~~

Figure ~~4~~4. ~~F~~our different SOC chemical structural complexity indexes (mean ± SE, n = 6) from solid-state ^{13}C CPMASNMR 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. ~~No significant differences were found.~~

Figure ~~5~~5. ~~T~~he factors regulating the SOC stock under (a) N enrichment,~~ and~~ (b) warming treatment and (c)interactive effects of N and warming. In the structural

970 equation model (SEM) analysis**,** black arrows represent significant positive pathways,

971 gray arrows represent significant negative pathways, and gray dashed arrows indicate

972 nonsignificant pathways. Values next to the arrows represent standardized effect sizes

973 with statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001). The thickness of

974 the arrow represents the standardized effect sizes. C-degrading enzyme indicate sum of

975 β-D-cellubiosidase (CB), α-glucosidase (AG), β-glucosidase (BG) and β-xylosidase

976 (XYL). Goodness-of-fit statistics for the model are shown as follows: (a), $\chi^2 = 4.53$, P

977 = 0.53, GFI =0.99, RMSEA < 0.001; (b), $\chi^2 = 4.47$, $P = 0.486$, GFI = 0.99, RMSEA <

978 0.001.

979 **Table 1.** Relative intensities (mean ± SE, n = 6) of different carbon chemical shifts from

980 solid-state ^{13}C CPMAS NMR spectra of soil samples from N and warming treatments.

981 ~~No significant differences were found.~~

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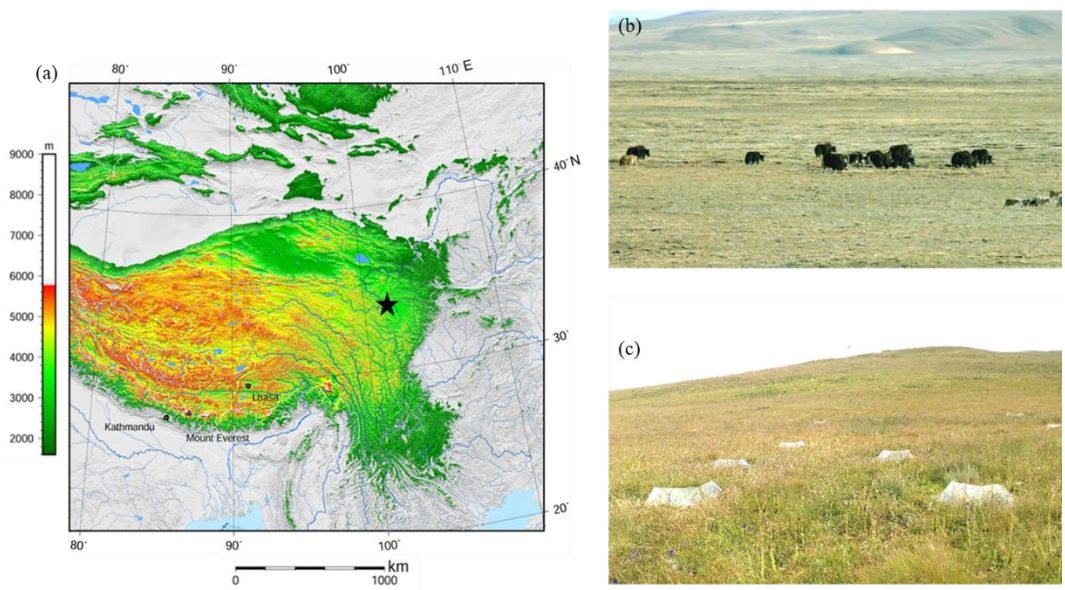
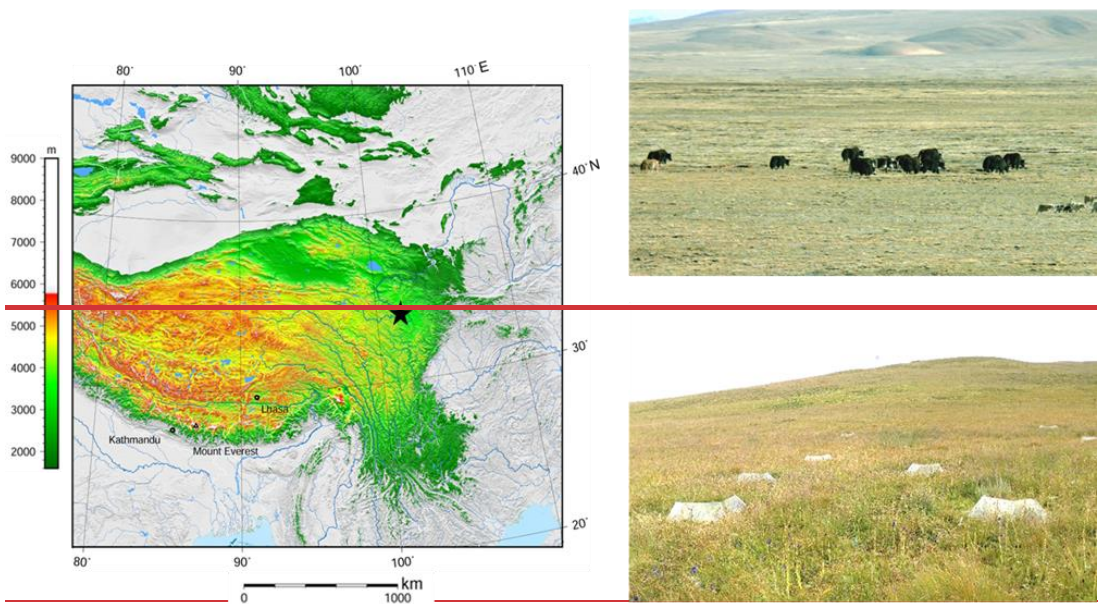
983 **Figure 1**

986   Note: the map (a) was cited from the Wikimedia Commons website (Tibet and

987   surrounding areas above 1600m, created using the Generic Mapping Tools)

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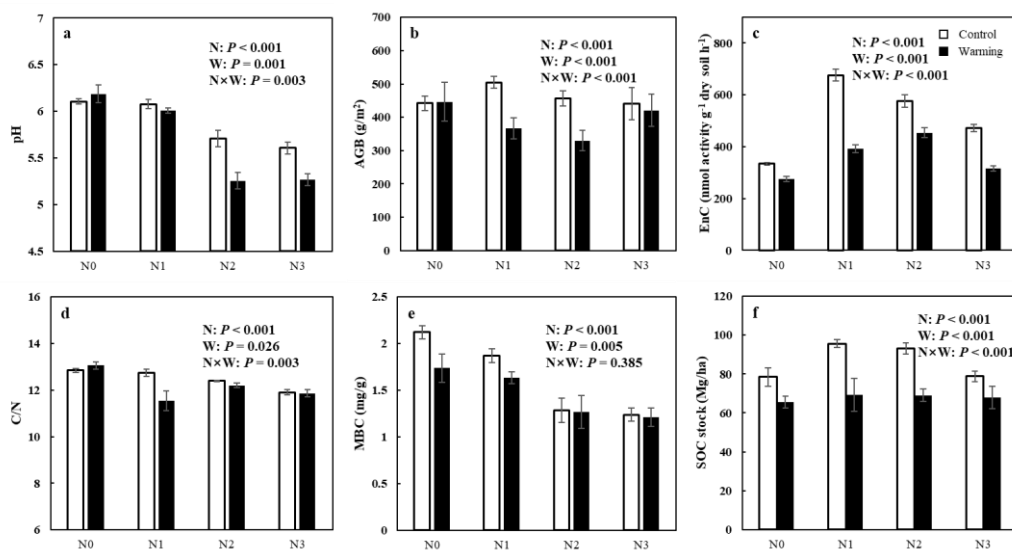
989 **Figure 2**

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Figure 3~~Figure 3~~

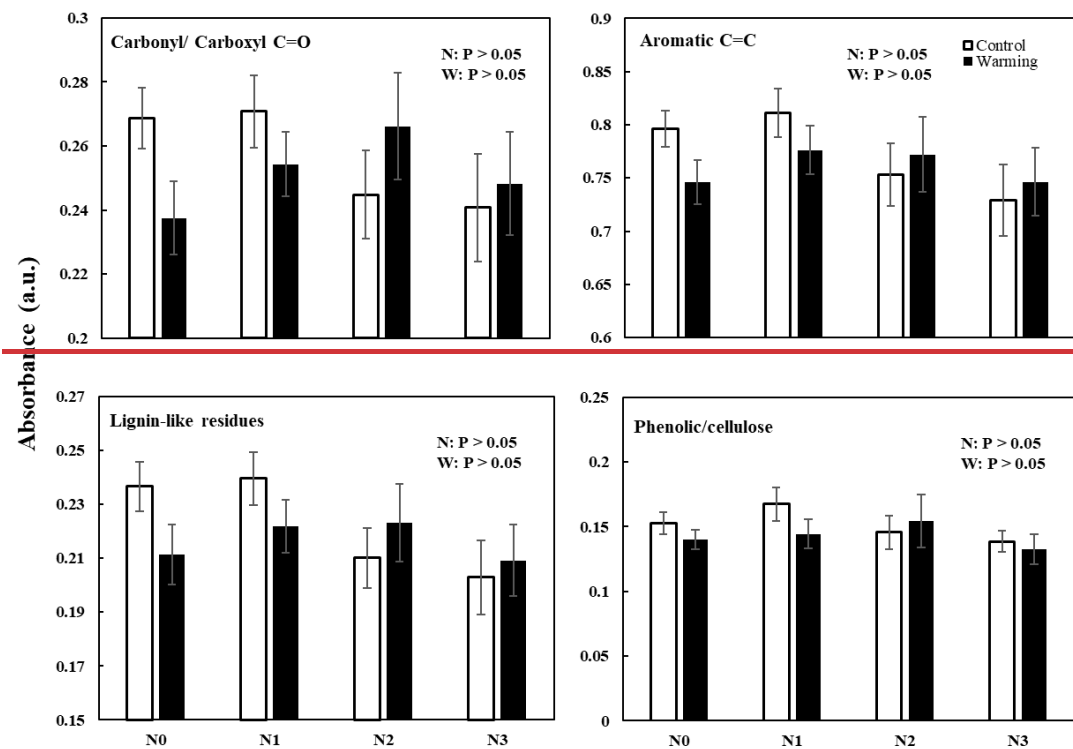
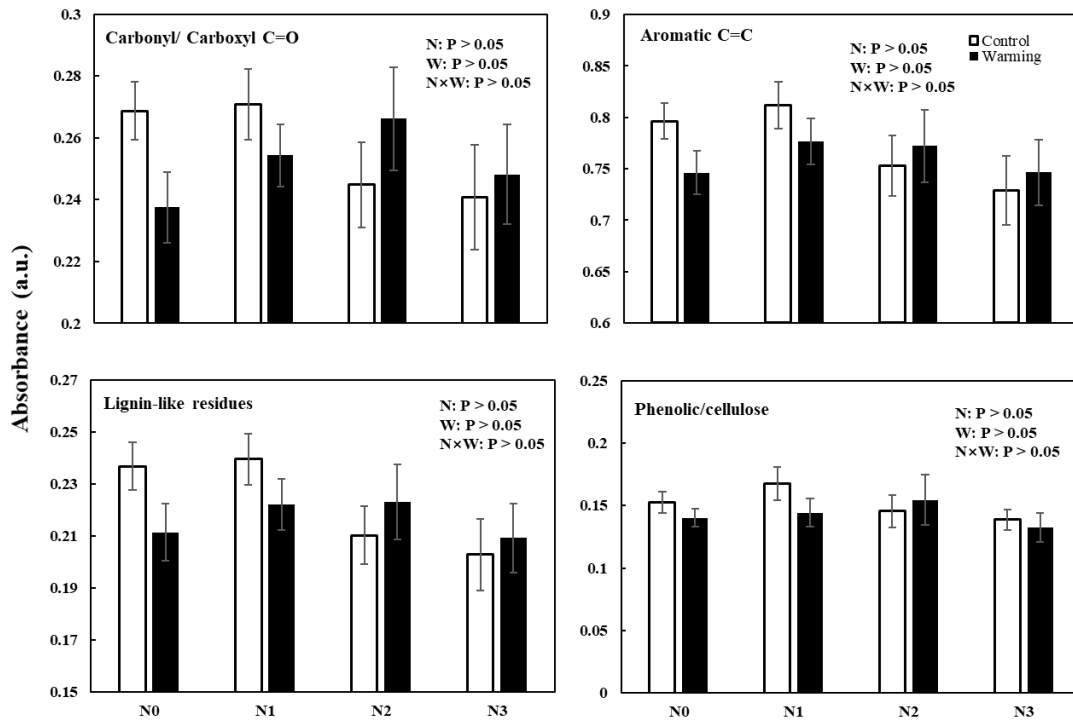
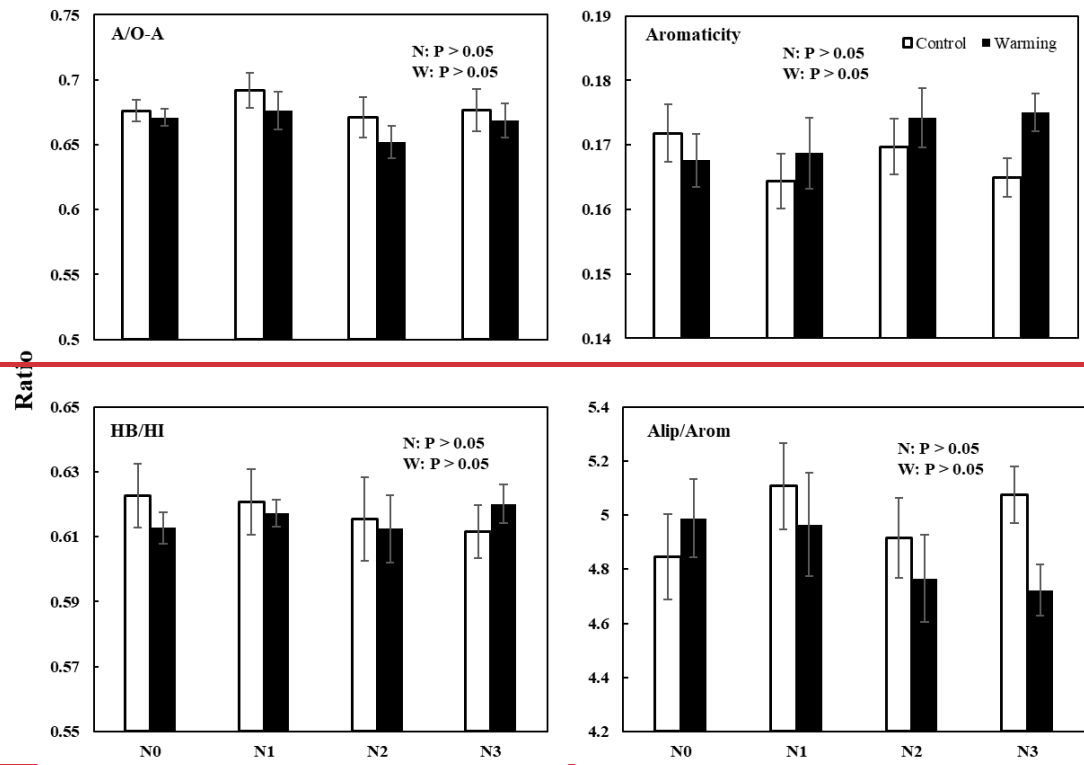
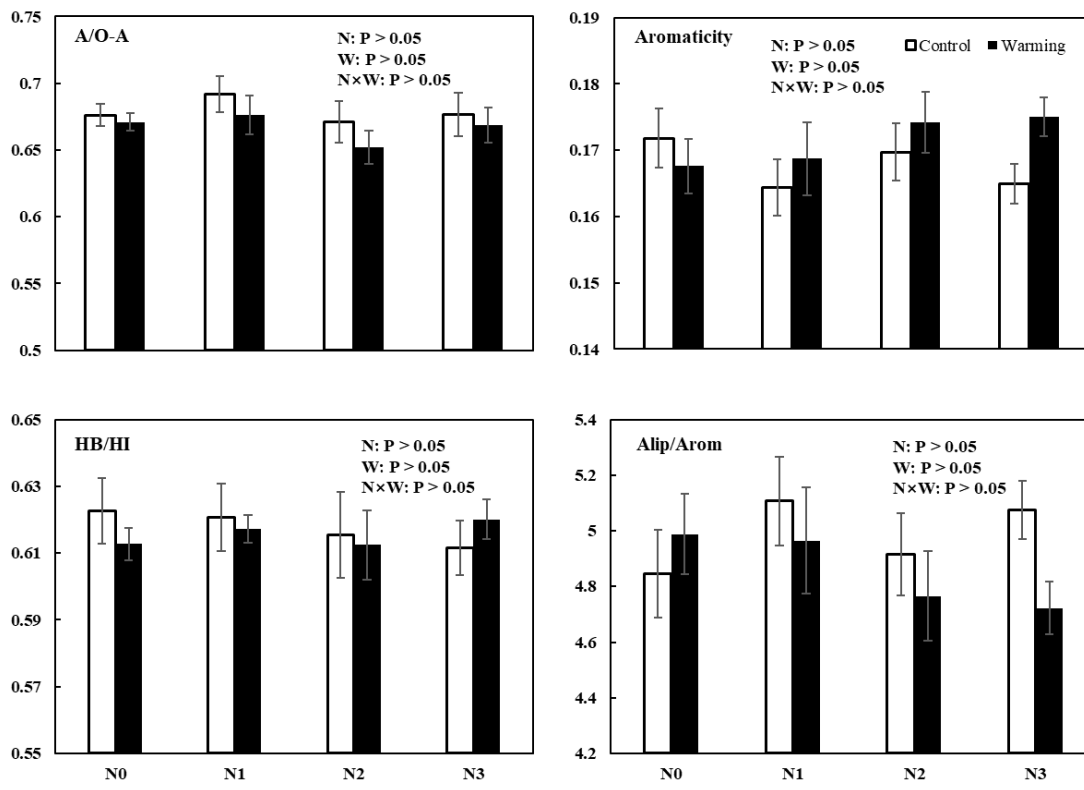



Figure 44

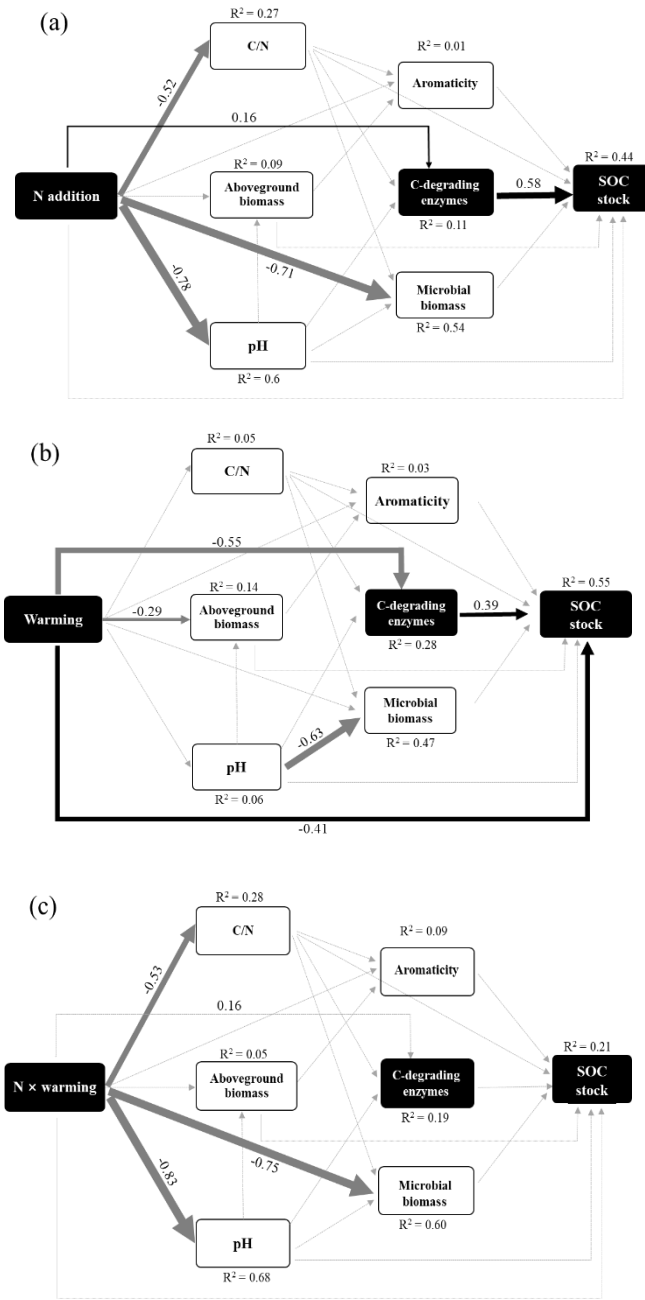
1001 **Figure 55**

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 Table 1

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