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1 **Nine years of warming and nitrogen addition in the Tibetan grassland**

2 **promoted loss of soil organic carbon but did not alter the bulk change**

3 **of chemical structure**

4 Huimin Sun[1, 2], Michael W.I. Schmidt[2], Jintao Li[1], Jinquan Li[1], Xiang Liu[3], Nicholas

5 O.E. Ofiti[2], Shurong Zhou[4], Ming Nie[1*]

6 1. Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering,

7   National Observations and Research Station for Wetland Ecosystems of the Yangtze Estuary,

8   Institute of Biodiversity Science and Institute of Eco-Chongming, School of Life Sciences, Fudan

9   University, Shanghai, 200433, China.

10 2. Department of Geography, University of Zurich, Zurich, Switzerland.

11 3. State Key Laboratory of Grassland Agro-Ecosystem, Institute of Innovation Ecology, Lanzhou

12   University, 222 Tianshui South Road, Lanzhou 730000, China.

13 4. Key Laboratory of Genetics and Germplasm Innovation of Tropical Special Forest Trees and

14   Ornamental Plants, Ministry of Education, College of Forestry, Hainan University, Haikou 570228,

15   China.

16 *Correspondence to*: Ming Nie (mnie@fudan.edu.cn)

17 **Abstract.** Understanding the changes in soil organic carbon (SOC) storage and

18 chemical stabilization dynamics is important for accurately predicting ecosystem C

19 sequestration and/or potential C loss, but the relevant information, especially for the

20 intervention of environmental controls on grassland soil is limited in Tibetan plateau

21 regions. Here we used a 9-year two-way factorial experiment involving warming with

22 open top chambers (+1.80 °C in the daytime and +0.77 °C in the nighttime at the soil

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23   surface) and multilevel nitrogen (N) enrichment treatments (0, 5, 10, and 15 g m^{-2} year^{-1}) in the Tibetan plateau to investigate the changes in SOC pool size and chemical

25   structure. 9-year warming treatment significantly decreased SOC stock in the Tibetan

26   grassland. We observed decreasing SOC concentrations which may be related to

27   changes in the C degrading enzymes. Surprisingly, the SOC molecular structure

28   remained unchanged in all N enrichment and warmed plots, suggesting that both

29   treatments had affected all forms of SOC, from simple and complex polymeric in a

30   similar way. Our results suggest that long-term warming stimulates soil C loss but no

31   preference in SOC loss with different chemical structure.

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

33   stabilization

34

1. Introduction

35

36   Soil organic matter is the largest organic carbon reservoir of near-surface terrestrial

37   ecosystem (Dlamini et al. 2016). Even subtle acceleration in SOC decomposition will

38   result in large CO_2 emissions (Davidson and Janssens 2006). So, knowledge of the

39   factors affecting SOC storage and decomposition is essential for understanding the

40   dynamically changing global C cycle. The influence of global warming on

41   decomposition of soil carbon has been well documented (Poeplau et al. 2017, Guan et

42   al. 2018, Ding et al. 2019b), but there remains considerable uncertainty in the potential

43   response of soil C dynamics to the rapid global increase in reactive nitrogen (N, coming

44   largely from agricultural fertilizers and fossil fuel combustion) as well as the combined

45   effects with warming (Liang and Balser 2012, Devaraju et al. 2015, Li et al. 2017). The

46   knowledge gap demonstrated a need to focus research on biological and

47   physicochemical controls of SOC stabilization and destabilization processes as a basis

48   for understanding causal relationships and key processes that determine pool sizes and

49   turnover rates of functional SOC pools (von Lützow and Kögel-Knabner 2009).

50   Soil warming experiments in the field have shown that warming generates a

51   considerable short-term soil C loss (Lu et al. 2013, Romero-Olivares et al. 2017). This

52   loss declines over time (e.g. > 2 years) (Romero-Olivares et al. 2017), although there is

53   evidence that it can continue for longer (e.g. > 20 years) (Melillo et al. 2017). Also,

54   indirect effects of warming on nutrient cycling (Pendall et al. 2004) or plant inputs

55   (Bradford et al. 2016) may have cascading effects on SOC quality and quantity (Lu et

56   al. 2013) and consequently on microbial decomposition of SOC, including recent plant-

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57 derived material (Hicks Pries et al. 2017) or older SOC (Vaughn and Torn 2019).

58 Because ecosystems in alpine meadow are normally N limited (Hobbie et al. 2002),

59 increased N released from decomposing SOC could stimulate plant productivity,

60 thereby increasing ecosystem C storage (Moscatelli et al. 2008). However, field

61 evidence suggests that soil microbial activity and biomass may also be N limited in

62 some C-rich ecosystems (Mack et al. 2004, Rinnan et al. 2007). Therefore, increased N

63 released from decomposition of SOC could further fuel microbial activity and decrease

64 soil C storage. Besides, according to the priming effect hypothesis, the increase in N

65 availability and labile C substrates promotes microbial C utilization, thereby increasing

66 the degradation of less decomposable SOC and leading to a negative effect on soil C

67 accumulation over the long term (Riggs and Hobbie 2016). However, it has been proven

68 difficult to quantify bulk SOC stocks changes and organic matter composition directly

69 (Sistla et al. 2013, Van Gestel et al. 2018). As alternatives, molecular-level techniques

70 can detect how temperature affects plant and soil organic matter, microbial growth and

71 their community composition under climate warming (Feng et al. 2008, Xue et al. 2016,

72 Pold et al. 2017).

73   Since the molecular structure of organic material has long been thought to determine

74 long-term decomposition rates in soil humic substances, solid-state CPMAS ^{13}C NMR

75 spectroscopy has been successfully applied in studies on changes of SOC chemical

76 structure during organic matter decomposition without any physical or chemical

77 destruction (Schmidt et al. 2011). However, because of the large number of variables

78 affecting a spectrum, it is extremely difficult to obtain a complete and fine molecular

79   structure from a single spectrum without additional knowledge obtained by other

80   spectroscopic techniques (Ferrari et al. 2011). So, we employed another complementary

81   molecular-level analysis called diffuse reflectance infrared Fourier transform (DRIFT)

82   spectroscopy, which is a useful method for the characterization of organic matter (Olk

83   et al. 2000) and humic substances (Mao et al. 2008, Francioso et al. 2009), to explore

84   potential shifts in SOC composition in response to warming and N enrichment. The

85   structure of SOC could be very complex but by combining both techniques (DRIFT and

86   solid-state ^{13}C NMR) complementary information could be obtained on aromatic and

87   aliphatic components (Ferrari et al. 2011).

88   Despite the importance of the response of SOC stocks to warming and N enrichment

89   in the intact ecosystem, this has not been assessed empirically in alpine meadows. This

90   knowledge gap is significant because the Tibetan Plateau stores a large C pool, with

91   36.6 Pg C stored in the top 3 m of the soil, accounting for 23.5% of China's total organic

92   soil-stored C and 2.5% of the global pool of soil C, which is of great importance in

93   regulating future global climate change and C emission (Genxu et al. 2002, Ding et al.

94   2019a). At the same time, the Tibetan Plateau has experienced climate warming at a

95   rate that is two times faster than that in other regions worldwide and is predicted to lead

96   to great soil C losses via microbial respiration in the future (Biskaborn et al. 2019). In

97   addition, during recent decades, the Tibetan Plateau has been subject to high levels of

98   N enrichment driven by agricultural activities (up to ~8.0 g m^{-2} y^{-1}) (Gao et al. 2007,

99   Bo et al. 2012, Zhang and Fu 2020) and atmospheric N deposition (1 g N m^{-2} y^{-1}) (Lü

100   and Tian 2007, Yu et al. 2019) with an annual rate of increase in deposition (0.053 g N

101    $m^{-2} y^{-1}$) (Liu et al. 2013, Wang et al. 2019b), and this kind of enrichment has been shown

102   to induce soil C loss and affect SOC stabilization in this typical N-constrained

103   ecosystem (Xiao et al. 2021).

104   Since temperature is one of the main drivers of the vegetation growth and

105   decomposition of organic matter, on-going climate change may alter biophysical

106   processes with consequences for ecosystem functioning, especially in highly sensitive

107   cold regions such as the alpine meadow on the Tibetan plateau (Piao et al. 2006, Yang

108   et al. 2008). However, how and to what extent physical stabilization of SOC shifts may

109   occur, and consequently SOC storage and C-climate feedback would respond to

110   warming and N enrichment in an alpine meadow ecosystem, remains largely unknown.

111   Here, we used soils from a 9-year experiment with a two-way factorial design involving

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

113   (0, 5, 10, and 15 $g\ m^{-2}\ y^{-1}$; marked as N0, N1, N2, and N3, respectively) (Liu et al. 2016)

114   on the Tibetan Plateau to examine the changes in the stock and molecular structure of

115   SOC.

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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. Alpine meadow is the main vegetation type in this area. 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). 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 and +0.77°C in the nighttime at the soil surface) and nitrogen (0, 5, 10, and 15 g m^{-2} y^{-1}, corresponding to N0, N1, N2, and N3, respectively) were manipulated, with six replicates per treatment (Liu et al., 2016). The 48 plots with roughly the same species

140 diversity and community structure were 5 × 5 m and were separated by 1 m from

141 adjacent edges. Additional details can be found in our previous studies (Sun et al., 2023).

142 Surface layer (0-10 cm) soils were collected from these 48 plots using a 4-cm-diameter

143 auger in August 2019. Then, the fresh soil samples were transported to the laboratory

144 on ice.

145 **2.3 Soil analysis**

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

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

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

149 suspension with a pH meter (PHS-3D, Rex, Shanghai, China). For soil organic carbon

150 (SOC) analysis, air-dried soil was ground and HCl-fumigated (Komada et al. 2008),

151 and then the SOC concentration was determined with an elemental analyzer

152 (FlashSmart, Thermo Fisher Scientific, USA). The SOC stocks (0-10 cm) are calculated

153 by multiplying the SOC concentration by the bulk density (Walter et al. 2016). At each

154 site, all plants in three plots (50 × 50 cm) were harvested and dried to determine the

155 aboveground biomass.

156 We measured the activity of four extracellular enzymes in the soil at an in situ pH

157 (Nie et al. 2013). The absorbance of the C degradation enzymes β-D-cellubiosidase

158 (CB), α-glucosidase (AG), β-glucosidase (BG) and β-xylosidase (XYL) were measured

159 using a Tecan infinite M200 microplate fluorometer (Grodig, Austria) with 365 nm

160 excitation and 460 nm emission filters. The activities were expressed in units of nmol

161    $h^{-1}\,g^{-1}$ dry soil. We combined CB, AG, BG and XYL into a C-degrading enzyme variable

162   (EnC).

163   **2.4 Bulk soil organic matter composition using DRIFT spectroscopy**

164   To characterize warming-induced changes in SOC composition, 6 mg of ground soil

165   sample was examined by diffuse reflectance infrared Fourier transform spectroscopy

166   (DRIFT). Mid-infrared spectra were recorded using a Bruker TENSOR 27 spectrometer

167   (Billerica, Massachusetts, USA) from 4000 to 400 cm^{-1} (average of 16 scans per sample

168   at 4 cm^{-1} resolution). Infrared absorption bands were represented by functional groups.

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

170   **2.5 SOC molecular structure examination using NMR spectroscopy**

171   The soil samples used for NMR spectroscopy analysis were pretreated using HF (2%)

172   to eliminate paramagnetic materials, e.g. ferric ion and manganese ion, that may affect

173   the NMR signals (Skjemstad et al. 1994, Schmidt et al. 1997, Mathers et al. 2002). The

174   solid-state NMR spectra (^{13}C-CP-MAS) were recorded on a Bruker AVANCE III

175   600 MHz instrument (Bruker Instrument Inc., Billerica, MA, USA). The acquisition

176   conditions were set at frequency of 75.5 MHz, with 20 kHz spectra width, 5 kHz

177   spinning speed, 2 ms contact time, and 2.5 s recycle time. The regions of 0–210 ppm

178   spectra were plotted.

179   We examined seven chemical shift regions to represent the main C functional groups

180   (Golchin et al. 1997, Sun et al. 2019). We report proportions of each chemical shift area

181   and calculated 4 ratios indicative for the characteristics of soil organic matter. The alkyl

182   C, the most persistent fraction of SOC, comes from original plant biopolymers (such as

183   cutin, suberin and waxes) or from metabolic products of soil microorganisms (Ussiri

184   and Johnson 2003). As these materials decompose, the relative abundance of O-alkyl C

185   in the litter materials decreases, and there is a progressive increase in alkyl C

186   (Bonanomi et al. 2013). Therefore, the ratio of alkyl C to O-alkyl C (A/O-A = C_{0-45}/C_{60-}

187    $_{90}$) is an index represents the extent of SOC decomposition, the higher this ratio, the

188   higher the decomposition degree of SOC (Wang et al. 2015). Aromaticity ($C_{110-165}/C_{0-}$

189    $_{165}$), was used to indicate the complexity of molecular structure (Dai et al. 2001). The

190   ratio of aliphatic C/aromatic C (Alip/Arom), $C_{0-110}/C_{110-165}$, also indicates the molecular

191   structure of soil C, with higher Alip/Arom means less aromatic nuclear structure in

192   humus. The hydrophobic C/hydrophilic C (HB/HI) ratio, $(C_{0-45} + C_{110-165})/(C_{45-}$

193    $_{110} + C_{165-210})$, was used to reflect the stability of soil aggregation (Spaccini et al. 2006,

194   Wang et al. 2010).

195   **2.6 Regulating factors of SOC indicated by structural equation model**

196   To access the direct and indirect effects of external factors on SOC stock, structural

197   equation modeling (SEM) was performed using the R package 'plspm' and

198   'piecewiseSEM' (Li et al. 2020). For this purpose, firstly, all data were tested for

199   normality using the Kolmogorov–Smirnov test, and the non-normal variables were log-

200   transformed. Secondly, we established a prior model based on prior knowledge of

201   effects and relationships among the driving factors. Finally, we selected the best model

202   based on overall goodness of fits, including the chi-square (χ^2) statistic, degrees of

203   freedom (df), whole-model P value, goodness of fit index, and the root-mean-square

204   error of approximation (Schermelleh-Engel et al. 2003).

205   **2.7 Statistical analysis**

206   All data are presented as the mean values of six field replicates. Any significant

207   differences in soil physicochemical properties among the different N enrichment levels

208   and warming treatments were identified by using two-way ANOVA followed by

209   Tukey's HSD post hoc test, with differences considered to be statistically significant at

210    $P < 0.05$. The statistical analysis was conducted using SPSS 13.0 and R version 3.5.1

211   (R Foundation for Statistical Computing, Vienna, Austria, 2013).

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3. Results

3.1 Bulk soil properties

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).

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 carbonyl/carboxyl C=O, and C=C aromatics compounds as well as lignin-like residues decreased (non-significant) after N enrichment, however, kept steady in warming plots (Figure 3 and S2).

The results of ^{13}C NMR spectroscopy indicated the relative abundance of different C components (Table 1, Figure 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 similar in the following

235   abundance order: O-alkyl C (mean 33%), followed by alkyl C (mean 22%), aromatic C

236   (mean 12%), N-alkyl C (11%), carbonyl C and di-O-alkyl C (mean 8%), and finally

237   phenolic C (mean 3%). The four indexes which can represent the extent of SOC

238   decomposition observed by NMR spectra also showed no significant difference under

239   all the treatments (Figure 4), suggesting that SOC showed a similar degradation state at

240   all N level enrichments and warming treatments.

241   **3.3 Factors driving the SOC stocks**

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

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

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

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

246   response to N enrichment and warming. Overall, the SEM explained 44% and 55% of

247   the variance in SOC stock driven by N enrichment and warming treatment, respectively.

248   In both patterns, C-degrading enzymes showed an important indirect factor in

249   regulating SOC stock. N enrichment had a positive effect on SOC stock by enhancing

250   enzyme activities. In contrast, warming had a negative effect on SOC stock by

251   inhibiting microbial enzymes. Besides, warming had a strong negative direct effect on

252   SOC stock (Figure 5b).

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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. 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 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.

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 Tibetan plateau stored large amounts

276 of SOC because of the permafrost soil, where limited C decomposition has led to the

277 accumulation of large SOC stocks (Hengl et al. 2014, Schuur et al. 2015). Previous

278 studies showed that the vulnerability of soils with large C stocks derives from the high

279 temperature sensitivity of C decomposition and biogeochemical restrictions on the

280 processes driving soil C inputs. Contrast with that, in soils with low initial C stocks,

281 small losses coming from accelerated decomposition induced by rising temperature

282 may be offset by concurrent increases in plant growth and soil C stabilization (Day et

283 al. 2008, Macias-Fauria et al. 2012, Crowther et al. 2015). However, in areas with larger

284 SOC stocks, accelerated decomposition exceeds the potential C accumulation of plant

285 growth, contributing to a significant C loss to the atmosphere.

286 **4.2. Effects of N enrichment and warming on SOC chemical compositions**

287 SOC chemical composition not only is controlled by the chemistry of the plant

288 materials input to the soil, but also by the microbial processing and degradation of SOC

289 (Baldock et al. 1992). Although N addition can stimulate plant growth and increase

290 litter fall, it can also accelerate or slow down microbial processing of plant residues,

291 thus altering the chemical composition of SOC (Wang et al. 2019a). Surprisingly we

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

293 and warmed plots (Figure 3, 4).

294 As the predominant chemical component of SOC across all treatments in our study,

295 O-alkyl C is mainly composed of carbohydrates, peptides and other labile organic

296 components derived from the fresh material, which could be preferentially degraded

297 compared with more resistant components such as alkyl C (Simpson and Simpson, 2012,

298   He et al. 2018). So, we hypothesized that this result could indicate that N and warming

299   may have the same impact on the input of fresh plants on the Tibetan plateau. Unlike

300   O-alkyl C, lipids represent the main source of alkyl C (aliphatic chains), which is

301   derived from original plant biopolymers. Lignin and tannin represent the main source

302   of aromatic C, together with phenolic C, mainly originating from lignin and amino acids

303   of peptides (Baldock et al 1992). All these components are more resistant to microbial

304   decomposition than labile O-alkyl C (Simpson and Simpson, 2012). Our results

305   suggested that the proportions of the stable SOC chemical structures remained the same

306   between the treatments, indicating the synchronous degradation of SOC. The alkyl/O-

307   alkyl ratio and aromaticity, normally regarded as the indicator of the relative stage of

308   SOC degradation and has been widely used as an indicator to reflect the complexity of

309   SOC chemical structure (Baldock et al. 1992), exhibited no change after 9-year N

310   enrichment and warming, suggesting that all N levels and warming treatments exerted

311   similar effects on the degradation of SOC and aromatic and complex molecular

312   structure (Zhang et al. 2013).

313   Infrared spectroscopy of SOC showed a statistically non-significant change in the

314   treated plots and the control plots, which were consistent with a previous study that

315   showed the 4.5 years of +4 °C whole-soil warming did not change the relative

316   abundance of carbonyl/carboxyl C=O, and C=C aromatics compounds in the surface

317   soils (above 20 cm) from a forest (Ofiti et al. 2021). Collectively, the above results

318   suggested that molecular structure of surface SOC may not be as sensitive to long-term

319   warming as we thought before (Atanassova and Doerr 2011, Chen et al. 2018a). Surface

320 SOC is dominated by recent (less transformed) plant-litter inputs, which is less

321 degraded and transformed than subsoil SOC (Ofiti et al. 2021). The lack of change in

322 plant- and microorganism-derived organic matter in the surface soil may be due to slight

323 drying and warming near the surface (Soong et al. 2021) which could have inhibited or

324 resulted in relatively less surface inputs. We noticed that warming significantly reduced

325 aboveground biomass in this study (Figure 2). Compared with labile SOC, stable SOC

326 can be more vulnerable to priming once microbes are provided with exogenous C

327 substrates. This high vulnerability of stable SOC to priming warrants more attention in

328 future studies on SOC cycling and global change (Zhang et al. 2022). Overall, stable

329 functional SOC molecular structure indicated that soil warming and N enrichment had

330 similarly affected easily decomposable and stabilized SOC of this C-rich grassland soil

331 despite the C loss.

332 **4.3 Regulating factors of SOC stock**

333 Our interpretation that prolonged warming could reduce SOC storage is further

334 supported by the simultaneous reduction of different C pool sizes characterized with

335 various chemical structural complexity with long-term warming. Many previous studies

336 have shown that microorganisms preferentially use the labile C pool for community

337 utilization and turnover after short-term warming (Melillo et al. 2002, Kirschbaum

338 2004). However, after the initial microbial assimilation of readily accessible SOC with

339 warming, soil microorganisms can acclimate to C starvation through utilization of

340 chemical less available C with continued warming (Chen et al. 2020). This

341 transformation in microbial preference of C substrates can be facilitated by changes in

342   C-degrading enzyme activities (Crowther and Bradford 2013).

343   Our results indicated that C-degrading enzymes could play a key role in regulating

344   soil C storage, which is in line with previous explanations for continued soil C loss with

345   long-term warming, such as shifts in microbial community and physiology (Melillo et

346   al. 2017, Metcalfe 2017), changes in microbial carbon use efficiency (Tucker et al.

347   2013), and increased microbial accessibility to litter and SOC (Doetterl et al. 2015,

348   Bailey et al. 2019), which are all closely related to changes in microbial C-degrading

349   enzyme activities. For example, warming decreased the abundance of lignin-derived

350   compounds but increased ligninase activity in a mixed temperate forest (Feng et al.

351   2008). Although only cellulase activity was measured in our study, a previous meta-

352   analysis study has shown significantly increasing ligninase activity after warming,

353   enhancing the evidence of microbial response to recalcitrant C pools and the evidence

354   of simultaneous loss of different C fractions after long-term warming (Chen et al.

355   2018b). Microbial utilization of recalcitrant C pools could substantially accelerate

356   overall soil C loss because depolymerization of these recalcitrant macromolecules

357   increases microbial accessibility to litter and SOC previously protected by recalcitrant

358   C pools (Schmidt et al. 2011, Lehmann and Kleber 2015, Paustian et al. 2016).

359

360

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 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.

Data availability

379 The data that support the findings of this study and those not presented within the article

380 and its Supplementary Information file are available from https://doi.org/

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Author contributions

383 M.N. developed the original ideas presented in the manuscript; H.S. performed the

384 overall analysis with the assistance from Jintao Li. and Jinquan Li, N. O.; X.L. and S.Z.

385 organized the field experiment; H.S., M.S. and M.N. wrote the first draft, and all authors

386 jointly revised the manuscript.

Competing interests

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

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Figure and table legends

Figure 1. Location of the studied sites.

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. 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. 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. he factors regulating the SOC stock under (a) N enrichment and (b) warming treatment. In the structural equation model (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 (*$P < 0.05$; **$P < 0.01$;

747 ***$P < 0.001$). The thickness of the arrow represents the standardized effect sizes. C-

748 degrading enzyme indicate sum of β-D-cellubiosidase (CB), α-glucosidase (AG), β-

749 glucosidase (BG) and β-xylosidase (XYL). Goodness-of-fit statistics for the model are

750 shown as follows: (a), $\chi^2 = 4.53$, $P = 0.53$, GFI =0.99, RMSEA < 0.001; (b), $\chi^2 = 4.47$,

751 $P = 0.486$, GFI = 0.99, RMSEA < 0.001.

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

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

754 No significant differences were found.
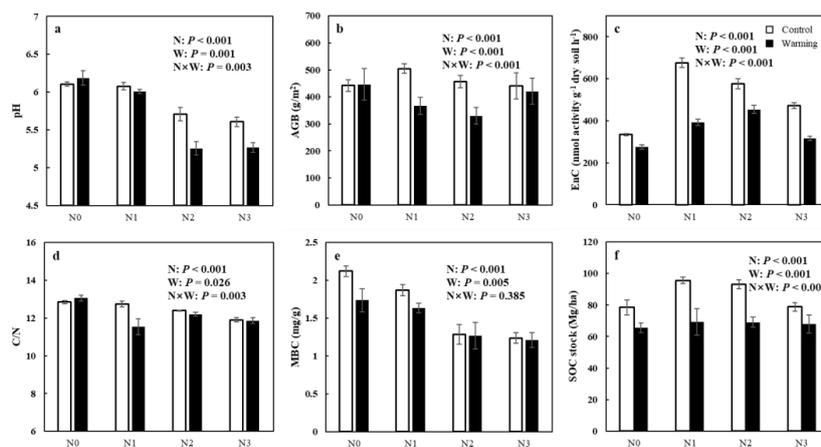
755

756 **Figure 1**
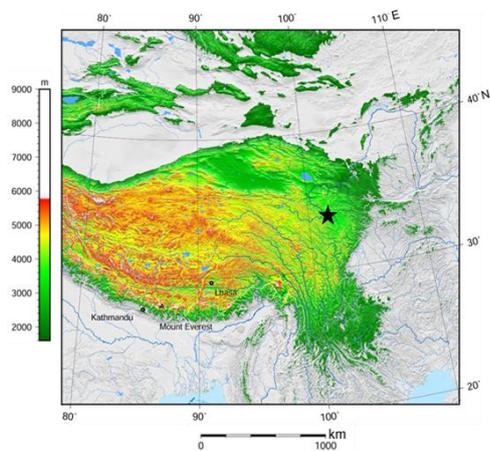
Note: the map was cited from the Wikimedia Commons website (Tibet and surrounding areas above 1600m, created using the Generic Mapping Tools)

Figure 2

765 **Figure 3**
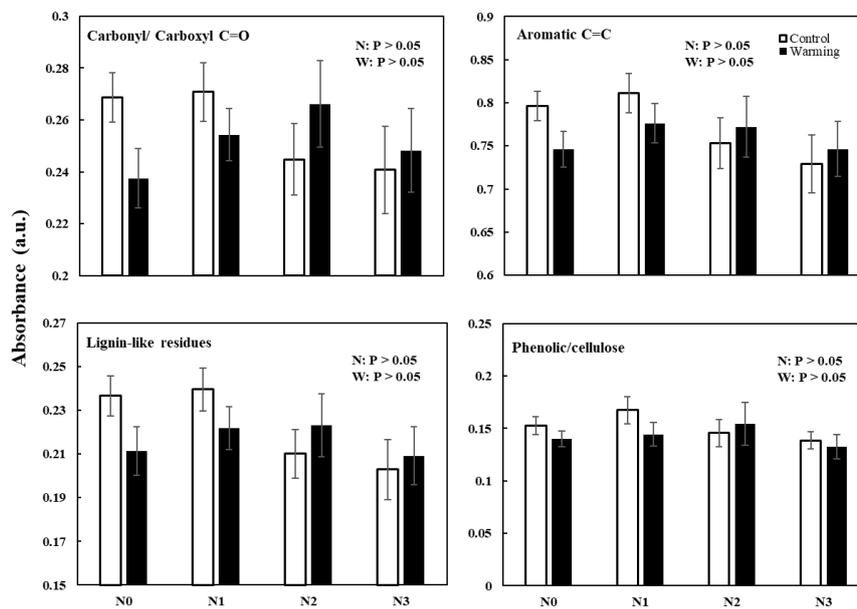

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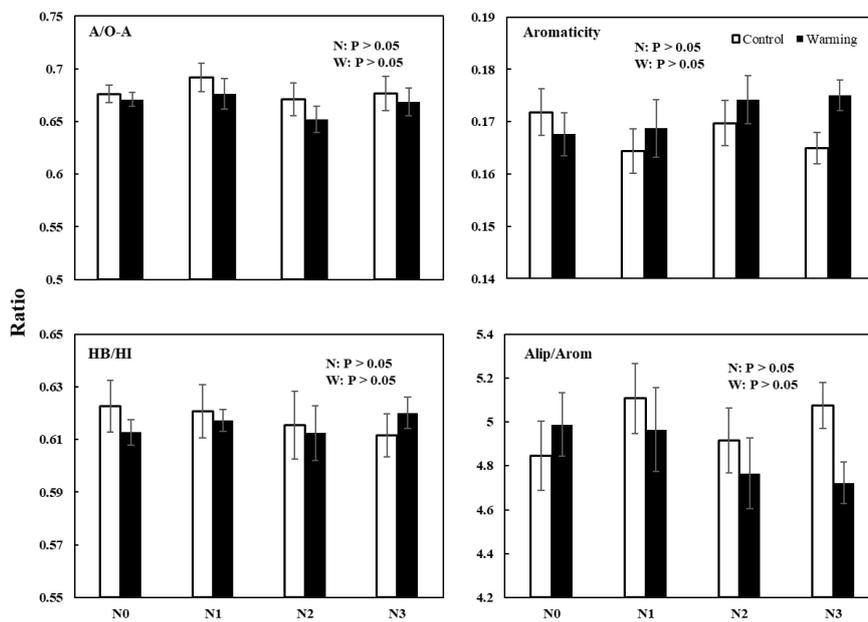767

768 **Figure 4**

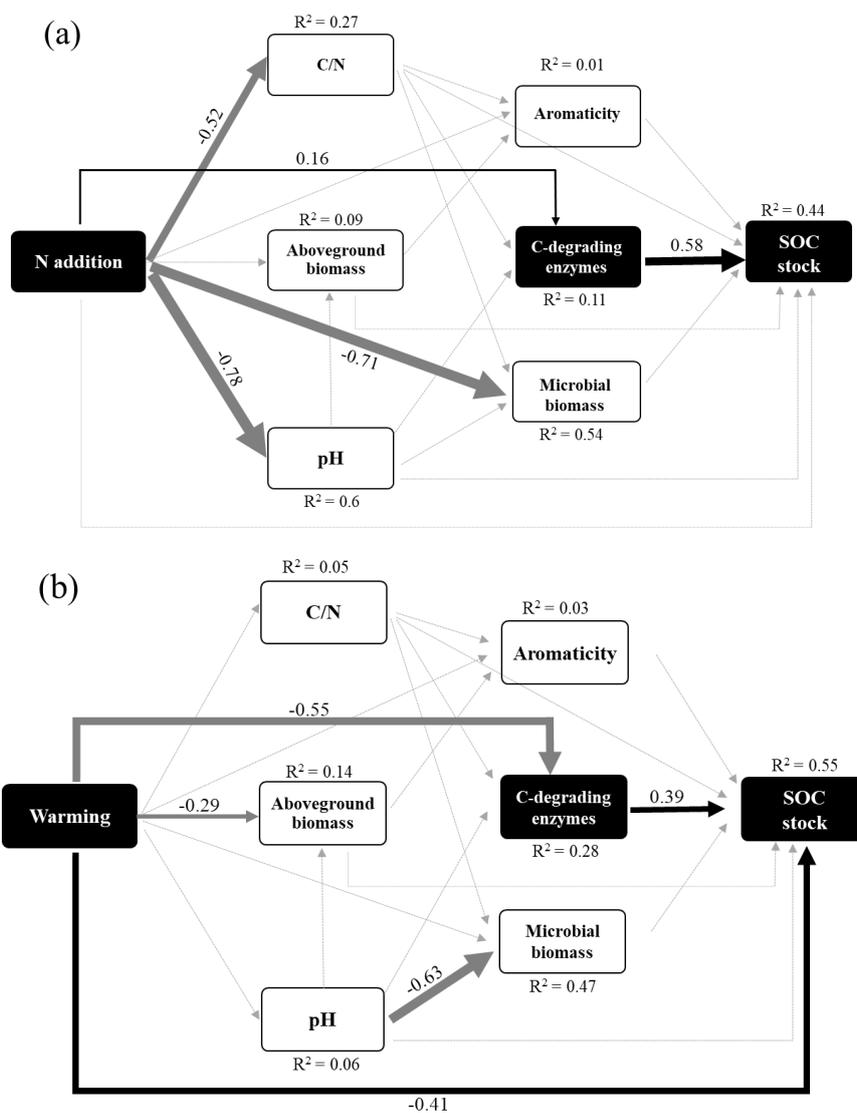**Figure 5**

776 **Table 1**

Chemical shifts		Treatment			
		N0	N1	N2	N3
¹³C 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

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