Article type: Primary Research Article

Title: Microbial metabolic response to winter warming stabilizes soil carbon

Running Title: Microbial responses to winter warming

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Abstract

Current consensus on global climate change predicts warming trends with more pronounced temperature changes in winter than summer in the Northern Hemisphere at high latitudes. Moderate increases in soil temperature are generally related to faster rates of soil organic carbon (SOC) decomposition in Northern ecosystems, but there is evidence that SOC stocks have remained remarkably stable or even increased on the Tibetan Plateau under these conditions. This intriguing observation points to altered soil microbial mediation of carbon-cycling feedbacks in this region that might be related to seasonal warming. This study investigated the unexplained SOC stabilization observed on Tibetan Plateau by quantifying microbial responses to experimental seasonal warming in a typical alpine meadow. Ecosystem respiration was reduced by 17-38% under winter warming compared with year-round warming or no warming and coincided with decreased abundances of fungi and functional genes that control labile and stable organic carbon decomposition. Compared with year-round warming, winter warming slowed macroaggregate turnover rates by 1.6 times, increased fine intra-aggregate particulate organic matter content by 75%, and increased carbon stabilized in microaggregates within stable macroaggregates by 56%. Larger bacterial 'necromass' (amino sugars) concentrations in soil under winter warming coincided with a 12% increase in carboxyl-C. These results indicate the enhanced physical preservation of SOC under winter warming and emphasize the role of soil microorganisms in aggregate life cycles. In summary, the divergent responses of SOC persistence in soils exposed to winter warming compared to year-round warming are explained by the slowing of

63 microbial decomposition but increasing physical protection of microbially-derived
64 organic compounds. Consequently, the soil microbial response to winter warming on
65 the Tibetan Plateau may cause negative feedbacks to global climate change and should
66 be considered in Earth system models.
67
68 **Keywords:** Winter warming; SOC stabilization; Microbial anabolism; Soil aggregate
69 turnover; carbon degradation genes; Microbial community
70

1. Introduction

Warming patterns caused by climate change are not equal across seasons (Piao et al., 2010; Suonan, Classen, Zhang, & He, 2017). Non-growing winter seasons experience larger temperature increases than summer growing seasons, especially in high latitude, temperature-sensitive areas (Piao et al., 2010; Kreyling et al., 2019). This 'asymmetric seasonal warming' (with the most significant temperature increase in winter) has been evident on the Tibetan Plateau over the last several decades (Liu & Chen, 2000; Li, Yang, Wang, Zhu, & Tang, 2010).

The Tibetan Plateau holds 30-40 Gt of soil organic carbon (SOC), which accounts for 2-3% of the global SOC stock (Shang et al., 2016). Increased losses of SOC to the atmosphere as CO_2 across this vast area, caused by accelerated microbial decomposition driven by warming, could constitute an important positive feedback to further climate change. The average temperature increase in the region is approximately twice the average global warming rate (Chen et al., 2013) and winter temperatures have risen by 0.3℃ per decade in the winter compared with 0.2℃ during the summer (Lu & Liu, 2010; Li, Yang, Wang, Zhu, & Tang, 2010). Despite this tangible warming trend, there is evidence that topsoil SOC stocks have remained remarkably stable (Yang et al., 2009; Liu et al., 2018; Chen, Feng, Yuan, & Biao, 2020) or even increased (Ding et al., 2017). This suggests that the underlying processes controlling the capacity of Tibetan grasslands to maintain SOC stocks under global warming are not fully understood and require investigation.

The regulation of SOC turnover is increasingly conceptualized as part of a dynamic

93 soil pore system, where the construction and destruction of pores controls SOC
94 availability to decomposing agents in the context of global change challenges
95 (Kravchenko et al., 2019; Franko and Schulze, 2020). Long-term physical protection of
96 SOC through aggregation afforded by the enmeshment of mineral particles by
97 biological agents is widely recognized (Tisdall and Oades, 1982; Six, Conant, Paul and
98 Paustian, 2002; Rillig and Mummey, 2006; Lehmann, Zheng, & Rillig, 2017). The
99 association of microbial 'necromass' (e.g. amino sugars from the residues of fungal and
100 bacterial cell walls) and mineral-associated fractions (Zhang, Amelung, Yuan, & Zech,
101 1998; Glaser, Millar, & Blum, 2006; Gunina et al., 2014) represents a direct link
102 between the stabilizing action of biota and microbial anabolism as a major contributor
103 to the stable SOC pool (Kallenbach, Frey, & Grandy, 2016) that is incorporated into
104 conceptual models and experiments (Liang, Schimel, & Jastrow, 2017; Sokol,
105 Sanderman, & Bradford, 2019; Gunina et al., 2014). Nevertheless, despite the
106 acknowledged stability of mineral-associated carbon, it is vulnerable to climate change.
107 Soil warming experiments, e.g. at the Harvard Forest (Pold, Grandy, Mellio, &
108 DeAngelis, 2017) and an annual grassland in California (Rillig, Wright, Shaw, & Field,
109 2002; Liang and Balser, 2012) revealed the depletion of mineral-associated organic
110 carbon. This is because increased temperature shifts the sorption-desorption balance
111 towards desorption (Conant et al., 2011) and decreases supplies of carbon as energy
112 source and organic substances that bind soil particles together (Giovannini, Lucchesi,
113 & Giachetti, 1988). However, the effects of soil warming in mesic regions may be
114 different from that at high latitudes in (semi)arid alpine regions, where warming is often

accompanied by severe summer droughts and frequent soil drying and rewetting, or freezing and thawing in winter and spring. Increasing intensity and duration of dry periods may progressively preserve SOC stocks (Borken and Matzner, 2009) through the formation of new and/or stronger organo-mineral interactions (Kaiser, Kleber, & Berhe, 2015) and may stabilize aggregates rather than disrupt them (Denef et al., 2001; Najera et al., 2020). Continuous warming led to increase of mineral-associated carbon along with suppressed soil respiration in a semiarid grassland (Bai et al., 2020). Microorganisms have specific adaptations depending on the local climate; for example, fungi in arid grasslands are less sensitive to drought than those in temperate grasslands (Ochoa-Hueso et al., 2018), and this will influence the amount and stability of the microbial necromass (Liang & Balser, 2012; Ding et al., 2019). For instance, in cold, wet alpine ecosystems, plants may take up more nitrogen in the warmer growing season, while microbial nitrogen pools increase later in the year (Jaeger III, Monson, Fisk, and Schmidt, 1999; Edwards & Jefferies, 2010; Kuzyakov & Xu, 2013) potentially alleviating competition for nitrogen in the cooler non-growing season. Thus, the tendency towards SOC stabilization observed in the warming Tibetan Plateau may be a product of the effects of climate on soil physical, biogeochemical and microbiological processes.

Manipulation experiments simulating climate change commonly apply uniform warming treatments (Ma, Zhao, Liu, & Liu, 2018; Jia et al., 2019), but this does not allow the potential ecological impacts of the differential seasonal warming observed in high latitude areas to be determined. To disentangle the processes caused by the

observed temperature increases at different times of year on the Tibetan Plateau, a specific winter warming treatment was imposed in addition to a year-round warming treatment that would allow the identification and isolation of physical (soil aggregate size), chemical (labile and stable organic carbon pools) and biological (community structure and carbon degradation genes) processes specifically related to winter warming in this study. Specifically, we tested the hypothesis that SOC stabilization on the Tibetan Plateau is caused by the effect of winter warming on the carbon cycling functions of the soil microbial community.

2. Materials and Methods

2.1 Experimental site and design

The study was conducted in an alpine meadow at the Damxung Grassland Station, located in the central, southern Tibetan Plateau (30°51′N and 90°05′E; 4333 m a.s.l.). The region has a semiarid continental climate (mean annual temperature 1.3°C, annual precipitation 477 mm) with 85% of precipitation occurring between June and August and the soil being frozen for 3 months, from November to January. The growing season is from late May to September. The shallow soil (0.3-0.5 m) is a Gelic Cambisol with 67% sand, 18% silt and 15% clay and a pH of 6.95 (Shi et al., 2006). The vegetation cover is approximately 30-50% *Kobresia pygmaea* C.B. Clarke var. *pygmaea*, *Carexmontis everestii* and *Stipa capillacea Keng* as dominant species (Supporting Information Table S1).

Field manipulations consisted of three warming treatments, including year-round

159 warming (YW), winter-warming (WW) and no warming (control). The field experiment
160 was established in 2010. In brief, 12 plots of three treatments with four replicates were
161 laid out in a completely randomized block design. Each plot was 1 m × 1 m. Open-top
162 chambers were used to generate artificially warmed conditions using the same methods
163 as the International Tundra Experiment that was established to study the responses of
164 alpine ecosystems to experimental warming (Birkemoe, Bergmann, Hasle, and
165 Klanderud, 2016). The open-top chambers were constructed using 3 mm thick
166 polycarbonate plastic (40 cm high x140 cm diameter base with a 100 cm diameter
167 aperture at the top). The open-top chambers were removed from the plots from late May
168 to late September each year in the WW treatment plots. Air and soil (5 cm) temperature
169 and soil moisture were recorded every 30 min (HOBO weather station, Onset Inc.
170 Bourne, MA, USA).
171
172 2.2 Ecosystem respiration (Reco)
173 Ecosystem respiration (Reco) was measured three times per month at 10-day intervals
174 from June to September in 2015 using a LI-8100 (LI-COR Bioscience, Lincoln, NE,
175 USA). PVC chambers (20 cm in diameter, 5 cm height) were inserted into the soil to a
176 depth of 3 cm with plants intact. Reco was measured from the linear rate of CO_2
177 accumulation within the sealed cylindrical headspaces. During the Reco measurement
178 process, PVC collars were covered by a removable lid that contained an opening with
179 a CO_2 sensor. After closing the lid, CO_2 monitoring within the cylindrical headspace
180 lasted for 1.5 min. Ecosystem CO_2 flux rates were calculated as a linear CO_2 increase

using the 1 s readings during the 1.5 min closure time, with the initial 15 s mixing time after lid closure discarded in a LI-8100 file viewer application software (Zong, Chai, Shi & Yang, 2018).

2.3 Soil sampling and analysis

Soil samples (0-20 cm depth) were taken using a soil corer (5 cm inner diameter) in June 2015. The soil samples were stored in airtight polypropylene bags and placed in a cool box at 4°C during transportation to the laboratory, where litter, roots and gravels were carefully removed by hand, and the soil was divided into several subsamples for different analyses.

Subsamples of fresh soil for analysis of ammonium-nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^--N) concentration were stored at 4°C for no longer than one week before analysis using an autoanalyzer (TRAACS-2000, Bran+Luebbe, Norderstedt, Germany) following 0.01 M KCL (1:10 w/v) extraction for 30 min. Subsamples for pH, SOC and total nitrogen analyses were air dried at room temperature. Inorganic carbon was removed from the soil samples using an HCl-fumigation. The SOC and total nitrogen contents of bulk and aggregate size fractions were determined by combustion using a Vario EL III Elemental Analyzer (Elementar, Langenselbold, Germany). Soil pH was measured with a pH meter after shaking the soil in deionized water (1:2.5 w/v) suspensions for 30 min. Subsamples for microbial community composition and functional gene (GeoChip) analysis were stored at -80°C.

203 2.4 Soil aggregate size fractionation

204 Soil aggregate size separation was performed using a three-step fractionation method
205 (Yan et al., 2012) (Supporting Information Figure S1). Bulk soil was separated into
206 three size fractions: macroaggregates (M, > 0.25 mm), free microaggregates (Fm, 0.25-
207 0.053 mm) and non-aggregated silt+clay (< 0.053 mm). The macroaggregates were
208 fractionated into coarse intra-aggregate particulate organic matter (Coarse iPOM, >
209 0.25 mm), microaggregates-within-macroaggregates (mM) and silt+clay fractions
210 using a microaggregate isolator (Six, Elliott, & Paustian, 2000). The mM fraction was
211 inverted in NaI solution (1.85 g cm^{-3}). The light fraction obtained by centrifugation was
212 non-occluded intra-aggregate POM inside macroaggregates but outside
213 microaggregates (Fine iPOM, 0.25-0.053 mm). The heavy fraction was dispersed by
214 shaking in 0.5% sodium hexametaphosphate solution for 18 h with 10 glass beads. The
215 dispersed samples were rinsed over a 53 μm sieve to isolate POM inside mM (mM-
216 POM) and silt+clay fractions inside mM (mM-silt +clay).

217

218 2.5 ^{13}C-CPMAS NMR

219 The composition of SOC was investigated by determining the relative abundances of
220 functional groups using solid-state ^{13}C cross polarization with magic-angle-spinning
221 (CPMAS) NMR. Soil samples were treated with 10% HF-HCL solution to concentrate
222 the organic matter and to remove paramagnetic minerals. ^{13}C-CPMAS NMR spectra
223 were acquired using an AVANCE III 400 WB spectrometer (Bruker BioSpin AG,
224 Fällanden, Switzerland) at 100 MHz for ^{13}C and 400 MHz for ^{1}H with a spinning rate

of 8 kHz, an acquisition of 20 ms, a recycle time of 3 s and contact time of 2 ms. The spectra were integrated into four chemical shift regions corresponding to alkyl C (0-50 ppm), O-alkyl C (50-110 ppm), aromatic C (110-160 ppm), carboxyl-C (160-190 ppm) functional groups (Skjemstad et al., 1994; Mathers and Xu, 2013). The NMR spectra were processed with MestReNova 5.3.1 software (Mestrelab Research S.L.Santiago de Compostela, Spain). The raw ^{13}C CPMAS NMR spectra are provided in the Supporting Information Figure S2.

2.6 Microbial residue analysis

Glucosamine, galactosamine and muramic acid were used as biomarkers for microbial residues ('necromass'). The compounds were extracted from air-dried soil samples ground to < 0.25 mm and analyzed as their aldononitrile derivatives followed the methods of Zhang and Amelung (1996) using myo-inositol as an internal standard. The derivatized compounds were separated on a gas chromatograph equipped with an HP-5 column (30 m × 0.25 mm × 0.25 μm) and quantified using a flame ionization detector (Agilent 6890A, Agilent Technologies, Littleton, CO, USA). Total microbial residues were estimated as the sum of glucosamine, galactosamine and muramic acid. To calculate bacterial residues, we assumed that all muramic acid was derived from bacteria:

$$\text{Bacterial residue C} = \text{muramic acid} \times 45 \quad (1)$$

where 45 is the conversion value to bacterial residue (Appuhn, Scheller, & Joergensen, 2006; van Groenigen et al., 2010).

247 Fungal residue was calculated by subtracting bacterial-derived glucosamine from
248 total glucosamine, assuming a 1:2 molar ratio for muramic acid and glucosamine in
249 bacterial cells (Engelking, Flessa, & Joergensen, 2007):

250 Fungal residue C = (mmol glucosamine - 2 × mmol muramic acid) × 179.2 × 9 (2)

251 where 179.2 is the molecular weight of glucosamine and 9 is the conversion factor of
252 fungal glucosamine to fungal residue (Joergensen & Wichern, 2008; van Groenigen et
253 al., 2010). Total microbial residue was estimated as the sum of fungal and bacterial
254 residue.

255

256 2.7 DNA extraction, quantitative PCR and amplicon sequencing

257 DNA was extracted from 0.25 g of well-mixed soil using the PowerSoil Isolation kit
258 (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.
259 The quality of the purified DNA was assessed based on the 260/280 nm and 260/230
260 nm absorbance ratios obtained, using a NanoDrop ND-1000 spectrophotometer
261 (NanoDrop Technologies Inc., Wilmington, DE, USA). The DNA was stored at -80°C
262 until use.

263 Bacterial and fungal abundances were determined by qPCR using the Power
264 SYBR Green PCR Master MIX (Biosystems, Warrington, UK) on an ABI 7500 Real-
265 Time PCR System (Applied Biosystems, Foster City, CA, USA). The following
266 primer sets were used: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 909R (5'-
267 CCCCGYCAATTCMTTTRAGT-3') for bacterial 16S rRNA gene abundance and
268 fungal ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 2043R (5′-

GCTGCGTTCTTCATCGATGC-3′). The following program was used for 16S rRNA gene amplification: initial denaturation at 98 °C for 2 min, followed by 34 cycles of 98 °C for 10 s, 56 °C for 60 s, and 72 °C for 30 s; for analysis of ITS1: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 58 °C for 30 s, and 72 °C for 1 min.

The V4-V5 region of 16S rRNA and the internal transcribed spacer (ITS1) region of the rRNA, were then amplified in order to construct bacterial and fungal community profiles, respectively, using high-throughput sequencing. The primers F515 (5′′-GTGCCAGCMGCCGCGC-3′) and R907 (5′-CCGTCAATTCMTTTRAGTTT-3′) were used to target the V4-V5 region of 16S rRNA because of the broad coverage to capture as wide diversity as possible (Yusoff et al., 2013). We used the ITS1 region for fungi due to its ability to discriminate against plant (Adams et al., 2013; Li et al., 2020). The following thermal program was used for amplification of 16S rRNA gene: initial denaturation at 95 ℃ for 3 min, followed by 27 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃for 45 s, and single extension at 72 ℃ for 10 min, and end at 4 ℃. The PCR amplification of ITS1 rRNA gene was performed as follows: initial denaturation at 95 ℃ for 3 min, followed by 35 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 45 s, and single extension at 72 ℃ for 10 min, and end at 4 ℃. PCR amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina

MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw sequences were subjected to quality control with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) primers were exactly matched allowing 2-nucleotide mismatching. Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1 (Edgar et al., 2011), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier Bayesian algorithm (Wang, Garrity, Tiedje and Cole, 2007) against the Silva database (https://www.arb-silva.de/) and the UNITE database (https://unite.ut.ee/) using confidence threshold of 0.7. We used a randomly selected subset of 24183 and 33197 sequences per sample for subsequent bacterial and fungal communities' analysis.

2.8 Microbial functional communities and intensities of carbon decomposition genes

Microbial functional communities and intensities of carbon degradation genes were determined using DNA hybridization performed using GeoChip 5.0 according to He et al. (2007) and Yang et al. (2013). Briefly, DNA samples were labeled with Cy-5

313 fluorescent dye using a random priming method and purified with the QIA quick
314 purification kit (Qiagen, Valencia, CA, USA). The DNA was dried in a SpeedVac
315 (ThermoSavant, Milford, MA, USA) at 45 °C for 45 min. GeoChip hybridization was
316 carried out at 42 °C for 16 h on a MAUI® hybridization station (BioMicro, Salt Lake
317 City, UT, USA). After hybridization, GeoChips were scanned by a NimbleGenMS200
318 scanner (Roche, Madison, WI, USA) at 633 nm, using a laser power and
319 photomultiplier tube gain of 100% and 75%, respectively. Raw GeoChip data was
320 analyzed using a data analysis pipeline as described previously (He et al., 2007; Yang
321 et al., 2013). The data were logarithmically transformed, and then divided by the mean
322 value of each slide. Spots that were flagged or with a signal-to-noise ratio less than 2.0
323 were considered poor in quality and removed from statistical analysis (He et al., 2007;
324 Yang et al., 2013).
325
326 2.9 Statistical analysis
327 SOC chemistry (functional groups), physical fractions and other variables were
328 analyzed using a one-way ANOVA with randomized block design by using SAS (SAS
329 Inc., 1996). Differences were considered significant at $p<0.05$, and a post hoc least
330 significant difference (LSD) test was carried out to compare differences between
331 warming treatments. The normal distribution of residues of the model were tested by
332 using “shapiro.test” function of the *stats* package in R v.3.2.1 (R Core Team, 2018).
333 The relationships between microbial residues and SOC physical and chemical fractions
334 were assessed with linear regression analyses using the “lm” function of the *vegan*

335 package in R v.3.2.1. (R Core Team, 2018).

336 Matrices of the pairwise taxonomic distance between bacterial, fungal and
337 functional microbial communities (Bray-Curtis) were constructed in R v.3.2.1 with the
338 *vegan* package (R Core Team, 2018). Non-metric multidimensional scaling (NMDS)
339 was used to assess changes in the bacterial, fungal and microbial functional
340 communities. Adonis analyses were further performed to confirm significant changes
341 in community structures under influence of warming and in any pair of samples. NMDS
342 and statistical analyses were performed in R v.3.2.1 with the *vegan* package (R Core
343 Team, 2018).

344 Linear discriminant analysis effect size (LEfSe) was performed to identify
345 significant differences in bacterial and fungal taxa among treatments (Segata et al.,
346 2011). The significant differences were analyzed by using the non-parametric factorial
347 Kruskal-Wallis (KW) sumrank test and then estimate the effect size of each feature of
348 each differentially abundant feature with linear discriminant analysis (http://
349 huttenhower.sph.harvard.edu/lefse/). A significance alpha of 0.05 and an effect size
350 threshold of 2 were used for all of the biomarkers evaluated.

351 To further investigate the relationship between Reco with environmental variables
352 and biogeochemical processes, partial least squares path modeling (PLS-PM) analysis
353 was performed. PLS-PM is a data analysis method for variables that can be summarized
354 by using latent variables, and the fact that linear relationships exist between latent
355 variables (Sanchez, 2013). Models with different structures are evaluated using the
356 goodness of fit (GOF) statistics, a measure of their overall predictive power, with GOF >

0.7 considered acceptable values (Sanchez, 2013). The models were constructed using the "inner plot" function of the R package (*plspm*) (R Core Team, 2018). The environmental drivers selected in the model were the main predictors according to their contribution for variation based on Random Forest analysis (% of increase of MSE). Based on the importance and the maximum explanation for variations of Reco, the environmental drivers selected in the model include soil water and temperature, carbon degradation genes, fungal residue, aboveground biomass, bacterial/fungal ratio and aggregates sizes fractions (non-aggregated silt and clay, mM, mM-silt and clay) (Supporting Information Figure S3).

3. Results

3.1 Soil temperature and moisture

Warming increased average topsoil temperature and decreased soil moisture compared with the ambient control soil (no warming) ($p<0.05$; Supplementary Figure S4). The average change in soil temperature for the duration of the experiment was +1.3 °C under YW and +1.0 °C under WW. The average change in soil moisture for the duration of the experiment was -4.7% under YW and -3.3% under WW. During the growing season (June to September), soil temperature under YW increased by 1.2 °C and soil moisture decreased by 4.5%. During the non-growing season, incorporating 'winter' (October to May), the soil temperature increased by 1.3°C under YW and 1.4°C under WW, and average soil moisture was decreased by 4.9% in both YW and WW treatments.

379 3.2 CO_2 fluxes (Reco) and SOC chemistry

380 The soils contained 1.8% organic carbon and 0.19% total nitrogen (C:N ratio 9.5) with
381 no significant differences between warming treatments (Figure 1a and b). Soil mineral
382 nitrogen concentrations [NO_3^--N + NH_4^+-N] under YW and WW were 60% and 25%
383 less, respectively, than in the control treatment (p<0.05; Figure 1c). Compared to the
384 control treatment, warming decreased the average Reco during the growing season
385 (June to September) by 17% and 38% under YW and WW, respectively, (p<0.05, Figure
386 1d). The CP-MAS NMR spectra of soils from all warming treatments had similar
387 proportions of major functional groups, with O-alkyl-C comprising about 40% of the
388 total in all treatments. Phenol-C was proportionately less in the YW plots than the
389 control. Carboxyl-C was proportionately larger under WW than YW or control (p<0.05,
390 Figure 1e).

391

392 3.3 SOC in aggregate size fractions

393 The warming treatments changed the percentage of aggregate-size fractions and their
394 SOC content. YW and WW increased the proportion and carbon contents of the non-
395 aggregated silt+clay fraction compared with control (p<0.05, Table 1). Soils under YW
396 had the smallest proportion of fine iPOM and associated carbon (p< 0.05, Table 1). The
397 proportion of mM increased by 22% under WW compared to the control (p<0.05, Table
398 1). The SOC in mM under WW was 1.57 times more than under YW (p<0.05, Table 1).
399 The proportions of mM-silt+clay and associated SOC were greater under WW than YW
400 (p<0.05, Table 1). Based on the ratio of fine iPOM to coarse iPOM within

401 macroaggregates, macroaggregate turnover rates were 1.6 and 1.2 times less in WW
402 compared to YW and control, respectively.
403
404 3.4 Abundance and composition of soil bacteria and fungi
405 Fungal abundance was reduced under WW compared to the control (p<0.05, Figure 2b).
406 The abundance of bacteria did not change (Figure 2a). The bacterial/fungal ratio under
407 WW was larger than YW (p<0.05, Figure 2c). NMDS of the sequencing data followed
408 by Adonis analysis indicated that the warming altered the bacterial and fungal
409 community composition (p<0.05; Supporting Information Figure S5). Further pairwise
410 comparison indicated that the microbial community was changed in response to
411 warming, and suggested that the difference was between the control and YW for the
412 bacterial community, and control and YW, and YW and WW, for the fungal community
413 (p<0.05; Supporting Information Table S2).
414 The outcome of the LEfSe analysis indicated that the relative abundances of
415 bacterial taxa from Acidobacteria (from phylum to class), Anaerolineae (from class to
416 genus) and Longimicrobiaceae (from family to genus) were larger in the unwarmed
417 control treatments relative to the warmed plots (p<0.05; Figure 3). Further pairwise
418 comparison revealed that the relative abundances of taxa from Acidobacteria,
419 Fibrobacteres and Gemmatimonadetes were smaller under YW treatment relative to
420 control (p<0.05; Supporting Information Figure S6). In contrast, abundant genera such
421 as Microbacterium, Friedmanniella, Sciscionella, Flavobacterium, Pedobacter,
422 Paenibacillus, Methylobacterium, Roseomonas and Sphingomonas were enriched in

YW plots ($p<0.05$; Supporting Information Figure S6). Regarding soil fungal taxa, YW increased Cystobasidiomycetes abundance (from class to genus) as compared to WW and control treatments ($p<0.05$; Figure 3). Mortierellomycota (from phyla to genus) were enriched under WW ($p<0.05$; Figure 3).

3.5 Microbial necromass carbon and linkage with soil aggregate turnover

Total residue carbon content was larger under WW than under YW ($p<0.05$, Figure 4e). Fungal necromass content in all soils were more than twice greater than bacterial necromass under all warming treatments, and there were no differences in fungal necromass. The bacterial necromass was 30.8% larger under WW than under YW ($p<0.05$, Figure 4d). Bacterial residue carbon increased with carbon associated with mM ($r=0.53$; $p=0.044$; Figure 5) and mM-silt+clay ($r=0.51$; $p=0.05$; Figure 5). Bacterial residue carbon had positive relationship with total mineral-associated organic carbon ($r=0.68$; $p=0.008$; Figure 5), but negative relationship with total POM ($r=0.88$; $p=0.001$; Figure 5).

3.6 Microbial functional communities, carbon decomposition genes and linkage with Reco

Both WW and YW treatments changed soil functional microbial community structure relative to the control, as indicated by NMDS and Adonis tests (Figure 6a; Supporting Information Table S2; $p<0.05$). Decreased abundances of total carbon degradation genes and specific genes associated with the decomposition of soluble storage

compounds, i.e. starch, and structural compounds from plant cell walls (hemicellulose, cellulose and lignin) and fungal cell walls (chitin) were observed in soils under WW (p<0.05; Figure 6b).

PLS path modeling of the water and temperature, aboveground biomass, edaphic (soil aggregate size fractions) and biological (fungal residues, bacterial/fungal ratio and carbon degradation genes) drivers of ecosystem respiration explained 96% of the Reco variance, and provided the best fit of the data (GOF of 0.71; Figure 7). The soil water content and temperature showed the largest effect on Reco variance via direct effect (path coefficient = -0.793). There were corresponding direct positive effects of carbon degradation genes on Reco variance (path coefficient = 0.687). The fungal residue and stable C fractions (silt+clay, mM and mM-silt+clay) both resulted in direct negative effect on Reco variance (path coefficient = -0.426 and -0.240).

4. Discussion

The combination of physical (soil aggregate fractions), chemical (^{13}C NMR-CPMAS) and microbiological (Reco, community composition and carbon degradation genes) analysis allowed us to explore the effects of different seasonal warming (year-round or asymmetric winter warming) compared to ambient conditions on SOC dynamics and stabilization in this study. Using this multi-proxy approach, we determined divergent responses of the soil microbial community to differential seasonal warming that may explain the observed SOC stabilization in Tibetan steppe grasslands under different seasonal warming. Two major mechanisms emerged related to the effects of the

467 treatments on microbial activity that affected the balance between SOC mineralization
468 and stabilization in the alpine meadow topsoils (Figure 8): (1) increased physical
469 protection of larger amounts of microbially-derived SOC in soil stable aggregates, and
470 (2) decreased microbial decomposition caused by changes in the microbial community
471 composition and the expression of carbon decomposition genes.
472
473 **4.1 Winter warming decreased microbial decomposition via changes in microbial**
474 **function and community structure**
475 Ecosystem respiration (Reco) in both year-round and asymmetric winter warming
476 treatments was substantially depressed, and to a much greater extent under the winter
477 warming treatment (Figure 1). This was related to the combination of increased soil
478 temperature and decreased soil moisture over several years (Figure 7). We did not
479 separate autotrophic and heterotrophic respiration as individual contributors to Reco in
480 this study, so cannot partition Reco between plants and soil microorganisms. However,
481 we determined that changing aboveground plant biomass in response to warming was
482 not a strong determining factor of Reco at the experimental site using PLS-PM analysis
483 (Figure 7), which agrees with our previous report (Zong et al., 2018), and there was no
484 difference in the much larger amounts of root biomass in response to warming
485 (Supporting Information, Table S4). A positive correlation between temperature
486 increase and rates of soil respiration after warming is widely reported (Rustad et al.,
487 2001; Melillo et al., 2002). In general, warming initially promotes microbial growth
488 which stimulates decomposition, driving significant CO_2 losses in the early stages of

many soil warming experiments (e.g. Rustad et al., 2001; Melillo et al., 2002; Karhu et al., 2014; García-Palacios et al., 2015; Romero-Olivares, Allision, & Treseder, 2017; Metcalfe, 2017). Soil moisture is an important factor regulating large-scale spatial patterns of Reco in Tibetan alpine grasslands (Geng et al., 2012) and inconsistent effects of experimental warming on soil CO_2 flux that are related to interannual fluctuations in rainfall have been reported in semi-arid steppe grasslands (Liu, Zhang, & Wan, 2009). Persistent moisture deficits in warmed soils may subsequently reduce soil CO_2 efflux and microbial biomass content (Liu, Zhang, & Wan, 2009; Quan et al., 2019). Thus, the reduction in Reco with warming may be due to a consequence of inhibition of microbial respiration or substrate supply as well as suppression of physiological activity by warming-induced soil water deficits (Niu et al., 2008).

Physiological stress and constraints on enzyme production due to reduced water availability (Allison and Treseder, 2008; Tiemann and Billings, 2011) are likely to have contributed strongly to the decrease of carbon decomposition genes in warmed soils (Figure 6) leading to the subsequent positive direct effect on Reco (Figure 7). This effect coincided with an increase in the intensities of stress response genes (Supporting Information, Figure S7), suggesting a potential tradeoff between stress tolerance versus resource acquisition as described by Malik et al. (2020). In support of this explanation, the abundance of functional genes involved in both labile and recalcitrant carbon cycling were significantly elevated by experimental increases in precipitation (Li et al., 2017). The larger reduction of carbon decomposition genes in the winter warming treatment (Figure 6), indicated that winter warming also inhibited the production of

511 carbon decomposition enzymes through a decrease of soil fungal abundance and altered
512 fungal community (Supporting Information,Table S3). Decreases in genes for labile and
513 recalcitrant carbon decomposition in response to soil drying caused by winter warming
514 could indicate a negative feedback mechanism that reduces SOC losses. Warming also
515 decreased the relative abundance of carbon decomposition genes in transplanted intact
516 soil monoliths moved between sites at different altitudes in Tibet (Yue et al., 2015). The
517 opposite was observed in irrigated arid soils where, for example, Li et al. (2017)
518 determined an increase in the abundance of functional genes involved in both labile and
519 recalcitrant carbon cycling after warming. This also contrasts with results from
520 temperate prairies, where microbial genes for complex organic compound degradation
521 were increased by warming (Feng et al., 2017). Our study therefore provides further
522 evidence that changes in the water cycle caused by climate change have the potential to
523 moderate warming-induced carbon losses and could even reverse the expected trend on
524 the Tibetan Plateau. This implies a more prominent role for soil moisture in regulating
525 SOC processing in the future than it has played in the past by enhancing the feedback
526 between soil warming and global climate change (Werner, Sanderman, & Melillo, 2020).
527

528 **4.2 Stronger physical protection of SOC under winter warming**

529 SOC associated with minerals is considered as one of the most fundamental long-term,
530 stable carbon pools (Six & Paustain 2014; Cotrufo et al., 2015). The mM fraction is
531 suggested as a robust indicator for management induced SOC changes over decadal
532 time scales (Six & Paustian, 2014). In this study, as with bulk SOC, there was no

difference between warming treatments in coarse iPOM-C concentrations or in the size distribution of macroaggregates (Table 1). However, macroaggregate turnover rates (based on the ratio of fine iPOM to coarse iPOM within macroaggregates; Six, Elliott, & Paustian, 2000) were up to 1.6 times less in the winter warming treatments compared to the control. So, in contrast to year-round warming which decreased soil aggregate stability, winter warming had slower macroaggregate turnover and increased carbon contents in smaller aggregate size fractions, indicating increased carbon stabilization via organo-mineral interactions and microaggregation (Table 1).

The enmeshing action of fungal mycelia in the formation of soil aggregates is widely recognized (Rillig & Mumme, 2006; Lehmann, Zheng, & Rillig, 2017). Rillig et al. (2002) reported that warming of an annual grassland in the USA decreased the water stability of soil aggregate and abundance of arbuscular mycorrhizal (AMF) hyphae (*Glomus* sp.). In this study, AMF were not measured directly, but a reduction in phylum *Glomeromycotina* abundance was determined under year-round warming (Supporting Information, Figure S6) indicating a mechanism for the reduction in macroaggregate stability under this treatment. The changes of the AMF fungi response to warming and their relationship to the soil aggregation represent a key focus for further study.

Microbial residues ('necromass') is an important constituent of stable SOC due to its tendency for sorption to mineral surfaces or protection within stable aggregates (Miltner, Bombach, Schmidt-Bruecken, & Kaestner, 2012; Liang, Schimel, & Jastrow, 2017; Kuzyakov & Mason-Jones, 2018), described as the 'entombing effect' by Liang, Schimel, & Jastrow (2017). Soil water content was up to ~5% less in the warmed

555 treatments in this study (Supporting Information, Figure S4). Soil drying can promote
556 the formation of new and/or stronger organo-mineral interactions with microbial
557 byproducts (e.g. amino sugars) (Kaiser, Kleber, & Berhe, 2015; Bai et al., 2020). The
558 importance of the contribution of microbial necromass to stable SOC pools has recently
559 been reassessed (Liang, Amelung, Lehmann, & Kästner, 2019). This was confirmed in
560 our study where up to half of total SOC was derived from amino sugars, and ~60% were
561 of fungal origin (Figure 5). Fungi tend to be more resistant to drought, while bacteria
562 are more resilient (de Vries et al., 2012) and can return close to control conditions upon
563 rewetting (Canarini, Kiaer, & Dijkstra, 2017). In our study, soils under winter warming
564 contained more of the mM and mM-silt+clay fraction than year-round warmed soils.
565 Bacterial residue carbon content was positively related to the carbon content of the mM
566 and mM-silt+clay fractions (Figure 5) revealing the enhanced physical preservation of
567 SOC under winter warming.
568
569 **4.3 The effects of winter warming on soil chemistry**
570 SOC dynamics associated with specific organic compounds can be sensitive indicators
571 of effects of warming because the temperature sensitivity of recalcitrant organic matter
572 increases with warming over time as the labile pool is depleted more rapidly by
573 increasing enzyme activity (Hartley & Ineson, 2008). Like Jia et al (2019), we
574 determined a decrease in plant-derived phenols from lignin and suberin (identified
575 herein as the phenol-C functional group by ^{13}C NMR) in topsoils after 5 years of
576 continuous warming experiment (YW treatment). A reduction in lignin content of plant

577 inputs was previously identified in warmed temperate grasslands suggesting a potential
578 mechanism (Henry, Cleland, Field, & Vitousek, 2005; Sanaullah et al., 2014). An
579 increased abundance of carboxyl-C was unique to winter-warmed soils (Figure 1).
580 Although carboxylic (fatty) acids are susceptible to rapid oxidation by soil
581 microorganisms, they have the potential for sequestration within the soil by complexing
582 with clay minerals and other forms of organic matter (Bull et al., 2000) suggesting a
583 route to enhanced physical protection (Figure 5 and Table 1) of plant and microbial
584 derived carbon. The analysis of lipid biomarkers has the potential to assign source of
585 the carboxyl-C in future analysis of warmed soils to complement the analysis of amino
586 sugars as indicators of soil microbial contributions to SOC.

587 Unlike winter warming, year-round warming caused a decline in total microbial
588 residues and bacterial residue carbon (Figure 4). This observation is similar to the
589 observed reduction of total microbial residues in soils in an annual grassland after 9-
590 years of continuous warming in the USA (Liang & Balser, 2012). Inorganic nitrogen
591 content was significantly less under year-round warming (Figure 1c) indicating nitrogen
592 limitation due to climate change in Tibetan alpine meadow ecosystems previously
593 recognized by Ding et al. (2019) that may be exacerbated by drought stress. A recent
594 large-scale field investigation along a 1,000 km transect in Tibetan Plateau revealed that
595 gross rates of N mineralization were positively associated with the soil moisture (Mao
596 et al., 2020). Microbial residues are comparatively rich in nitrogen (Cotrufo et al., 2015)
597 and fungal residues (derived from chitin) are considered to be more persistent than
598 bacterial residues (derived from peptidoglycan; Six, Frey, Thiet, & Batten, 2006; Ding

et al., 2019) making the latter more susceptible to nitrogen mining during the growing season. Jaeger III et al. (1999) reported the transfer of microbial nitrogen to plant available pools was observed in the early growing season in an alpine ecosystem. Most organic nitrogen is associated with clay-sized particles where physicochemical interactions may limit the accessibility of N-containing compounds (Jilling et al., 2018). From an alternative perspective, the greater aggregate turnover under year-round warming may result in potential destabilization of bacterial residues from clay-silt fractions (Table 1 and Figure 5) due to microbial exploitation of organic nitrogen sources under conditions of low mineral-N availability (Figure 1).

5. Conclusions

With respect to other grassland ecosystems, alpine grassland systems account for more than 40% of the Tibetan Plateau area and are considered to be particularly sensitive to climatic change. Compared to year-round warming, winter warming reduced macroaggregate turnover, increased bacterial residues (necromass) and increased mM, an aggregate size fraction that is closely related to the long-term physical protection of SOC of microbial origin. Winter warming decreased the activity of carbon decomposition genes to a greater extent than year-round warming.

The deliberate isolation of winter warming from year-round warming in our field experiment allowed us to disentangle its effects in situ, to reveal potential mechanisms for observed SOC stabilization as a climate-C feedback. The fact that this was different from the year-round warming treatments suggests that summer can weaken the effects

of winter warming, and further study is required to determine the relative importance of winter versus summer warming in controlling SOC protection mechanisms. In this context, it would be valuable to establish experiments in which year-round warming treatments also reflect the actual and predicted differences in the magnitudes of climate warming in different seasons, with greater absolute warming in winter on the Tibetan Plateau.

Overall, our findings demonstrate that understanding the effects of warming at differences times of year on SOM protection mechanisms is critically important for predicting whether SOM will be lost or gained in response to climate change.

Acknowledgements

This study was supported by the National Key R& Program of China (grant no. 2017YFA0604803) and National Natural Science Foundation of China (grant no. 31770560; 32071629; 41703079). We thank for the support of the Government Program of Competitive Growth of Kazan Federal University and the "RUDN University program 5-100".

Conflict of interest

The authors declare no conflict of interest

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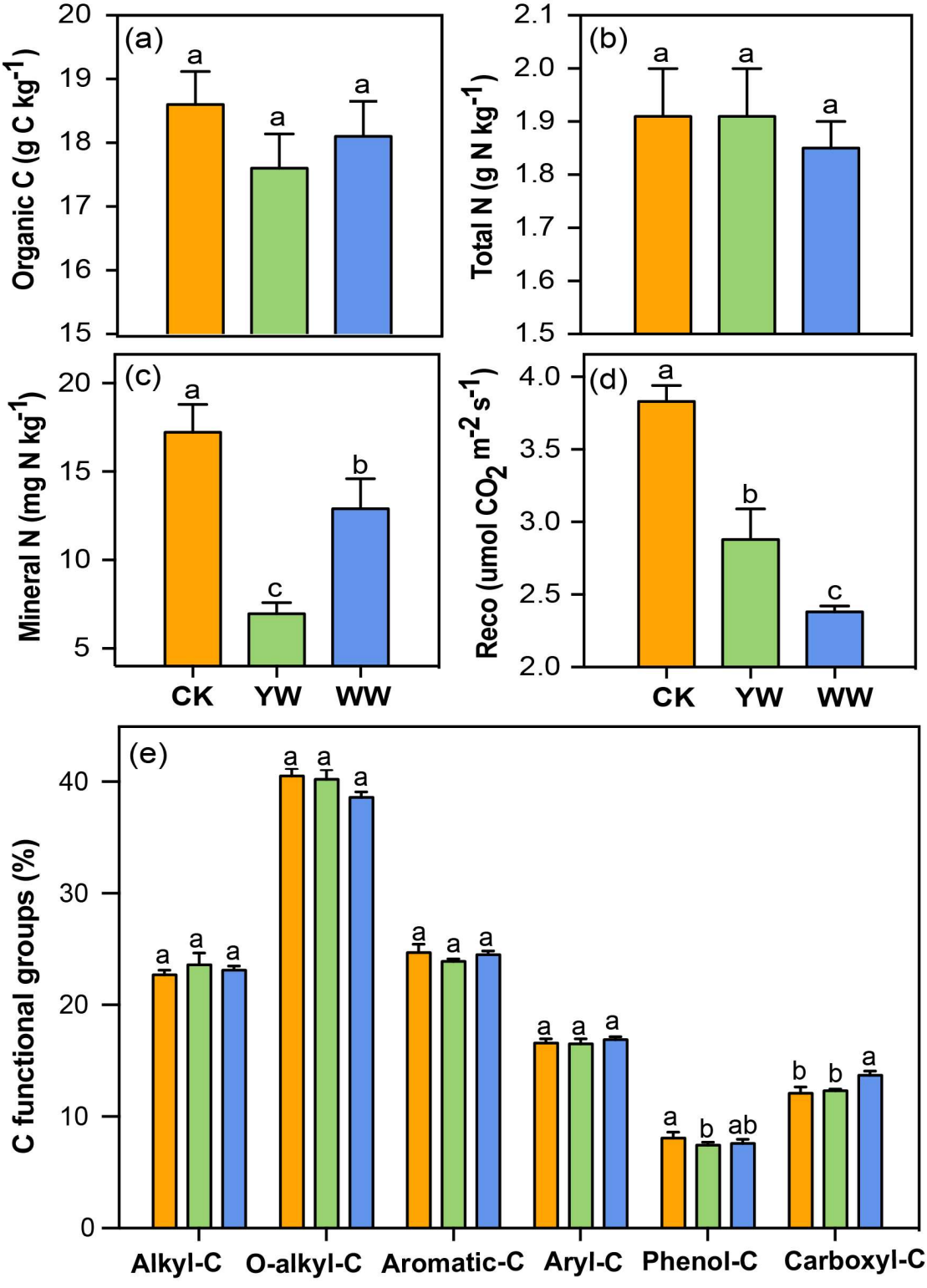
989 **Figures**



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991

992 **Figure 1 Effects of warming on soil chemistry, organic matter functional groups**
993 **and C flux (Reco)**

994 Lowercase letters indicate significant differences between treatments; error bars

995 indicate standard error of the mean (n=4). CK: control; YW: year-round warming; WW:
996 winter warming.
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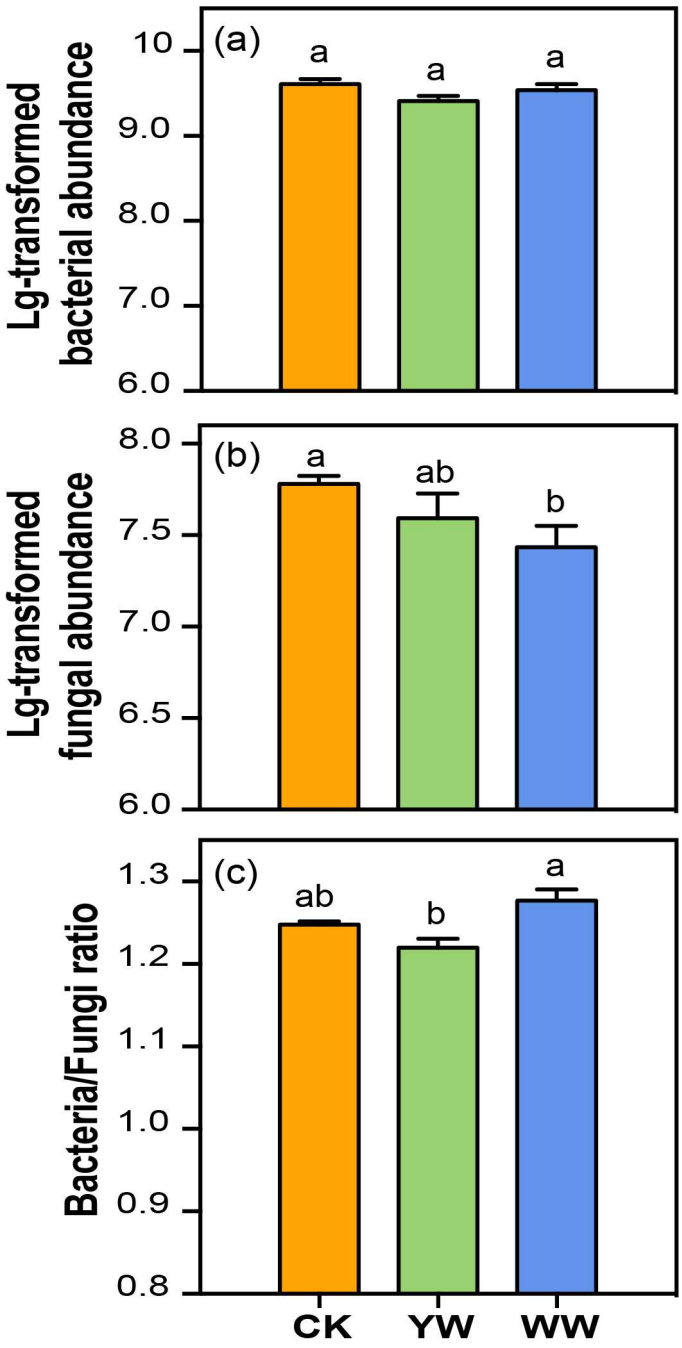

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999 **Figure 2 Effects of soil warming on (a) bacterial and (b) fungal abundances and (c)**

1000 **bacteria/fungi ratio**

1001 Lowercase letters indicate significant differences between treatments; error bars

1002 indicate standard error of the mean (n=4). CK: control; YW: year-round warming; WW:

1003 winter warming.

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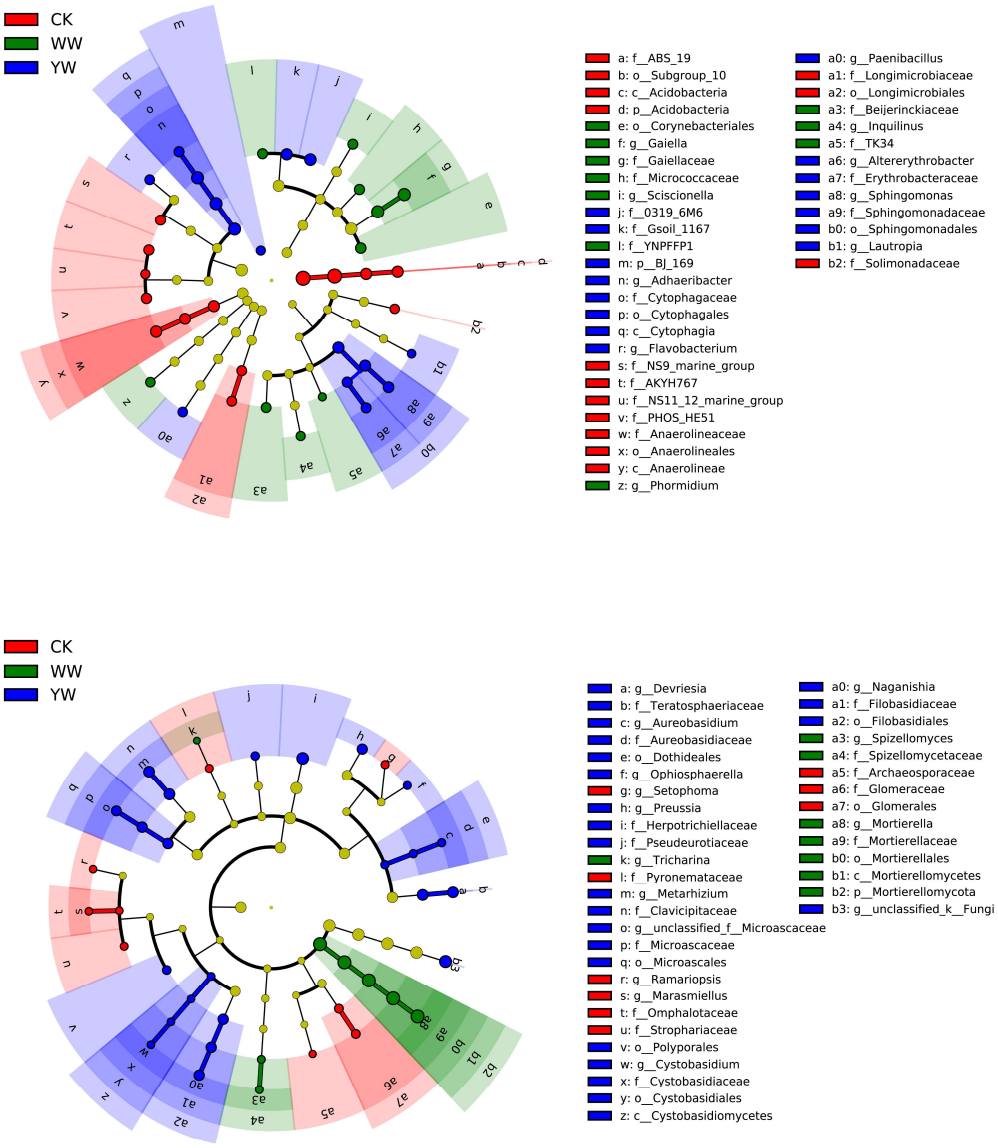


Figure 3 LEfSe analysis of significantly abundant taxa of bacteria (a) and fungi (b) in soil microbial communities under different treatments

In the evolutionary branch diagram, the circle radiating from inside to outside represents the classification level from the phylum to the genus. Each small circle at a different classification level represents a classification at that level. Differently colored nodes indicate the taxa that are significantly enriched in the corresponding group (red indicating control, green indicating winter warming and blue indicating year-round warming), and yellow nodes indicate taxa that have no significant difference. The threshold on the logarithmic LDA score was 2.0.

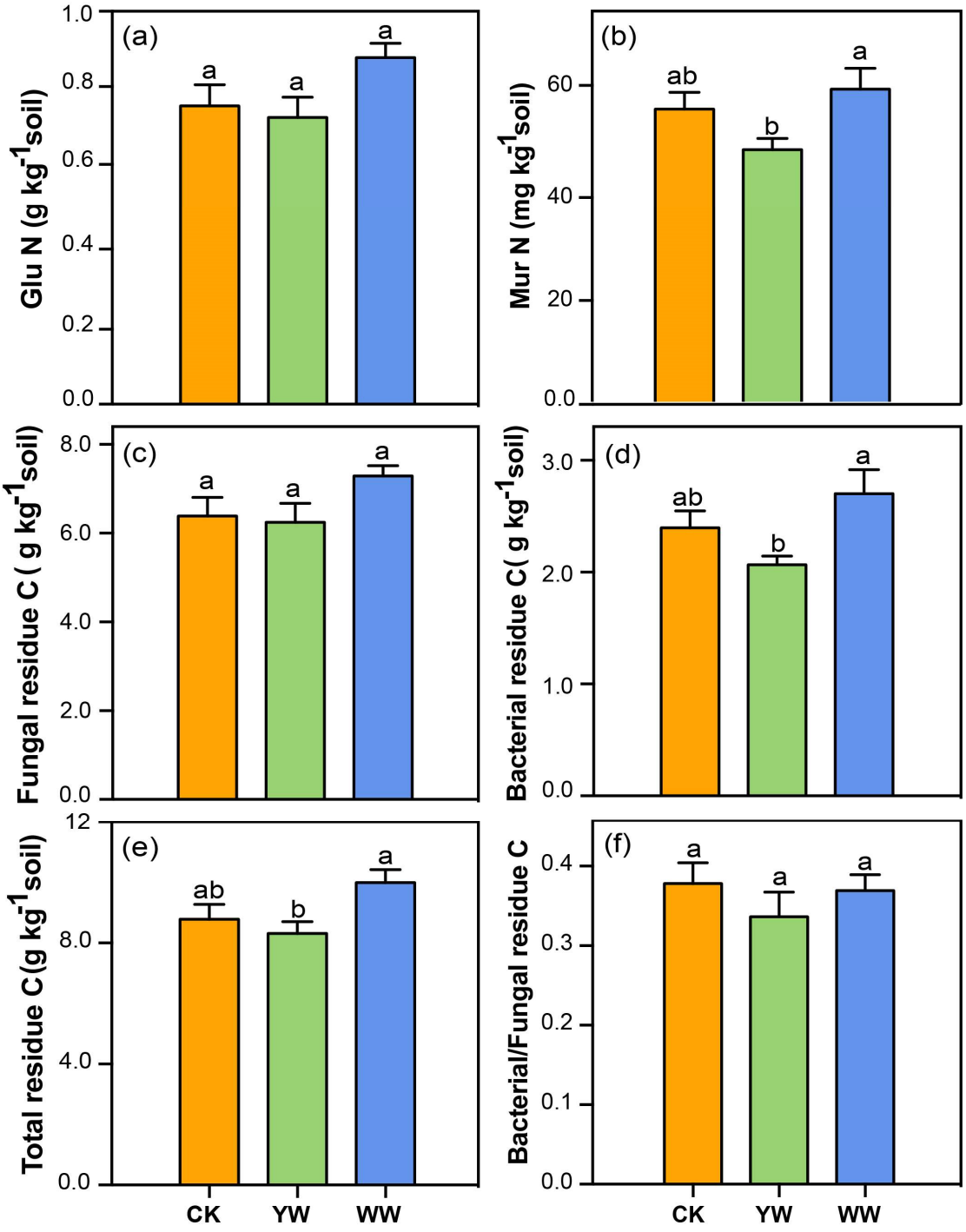
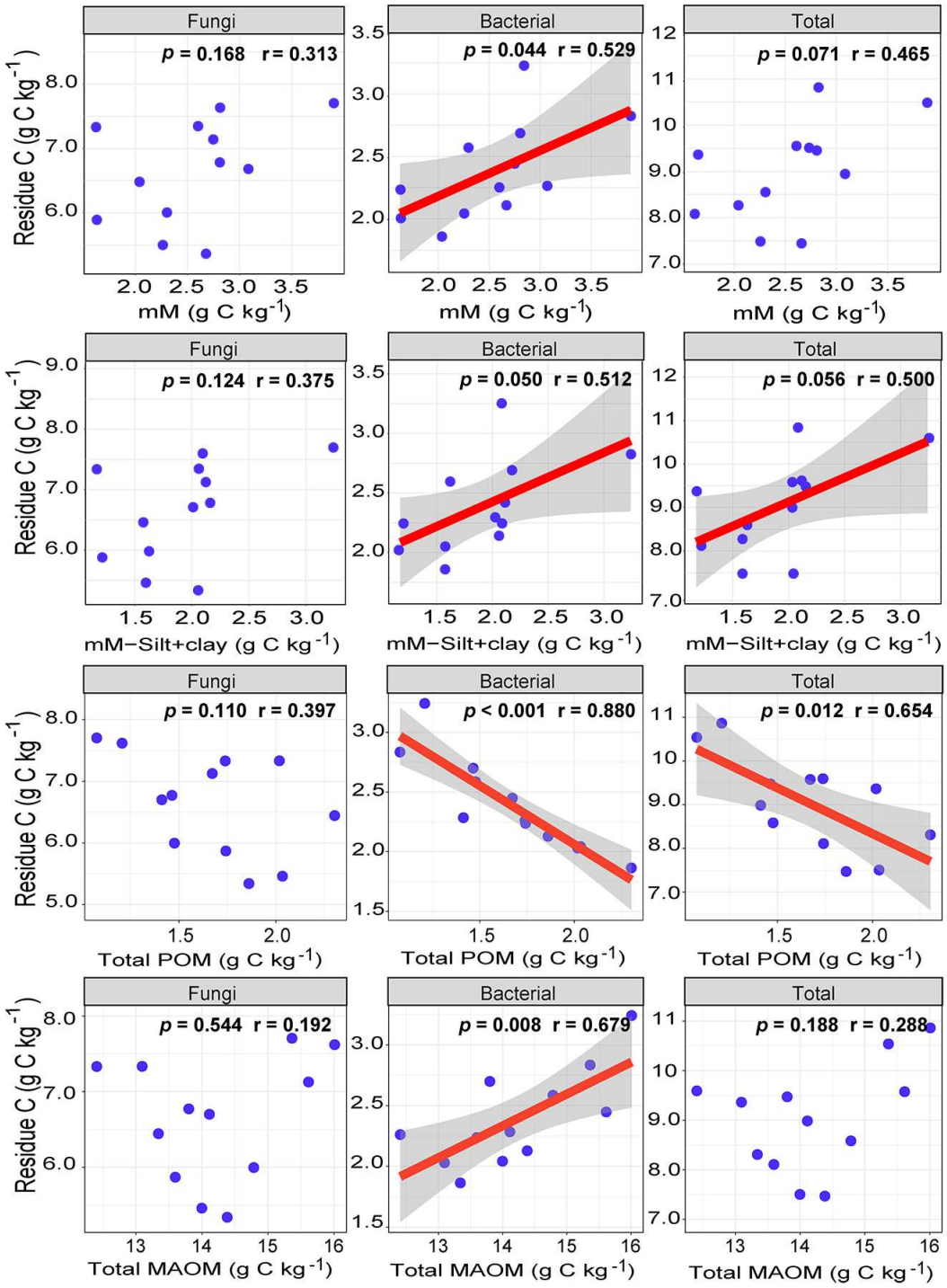


Figure 4 Effect of soil warming on amino sugar abundances and bacterial and fungal residue derived carbon

Lowercase letters indicate significant differences among treatments; error bars indicate standard error of the mean (n=4). CK: control; YW: year-round warming; WW: winter warming.



1023

1024 **Figure 5 Relationships between bacterial and fungal residue carbon and soil**

1025 **aggregate fractions**

1026 The red fitted lines are from linear regression. Only significant fitted lines are

1027 displayed on the graphs. Shaded areas show 95% confidence interval of the fit.

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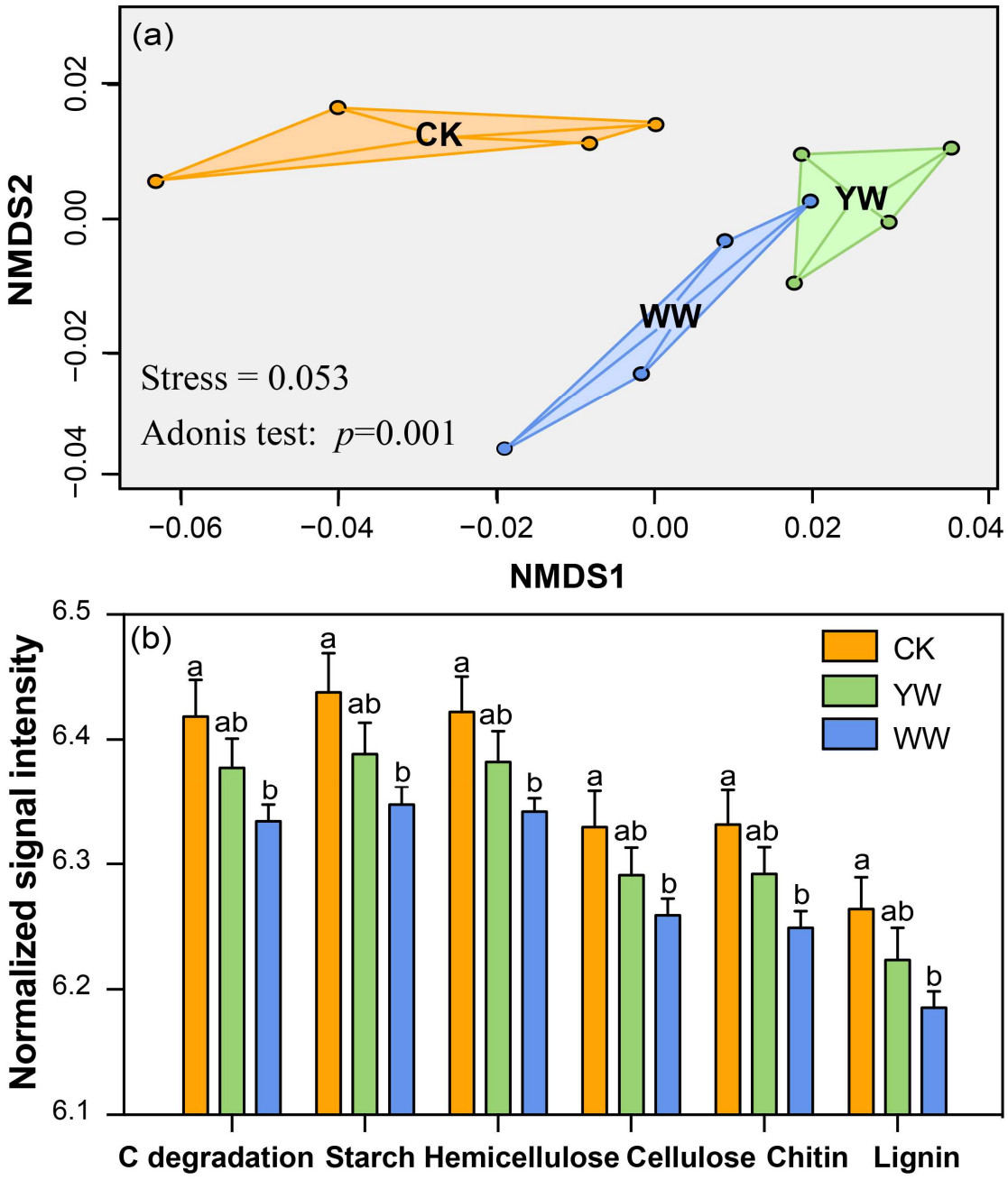

Figure 6 Effects of soil warming on functional microbial communities (a) and carbon degradation genes (b)

Lowercase letters indicate significant differences between treatments; error bars indicate standard error of the mean (n=4). CK: control; YW: year-round warming; WW: winter warming.

The functional microbial communities were ordinated by non-metric multidimensional scaling (NMDS) analysis. The stress value for the plot was < 0.06 which indicates that these data were well represented by the two-dimensional representation.

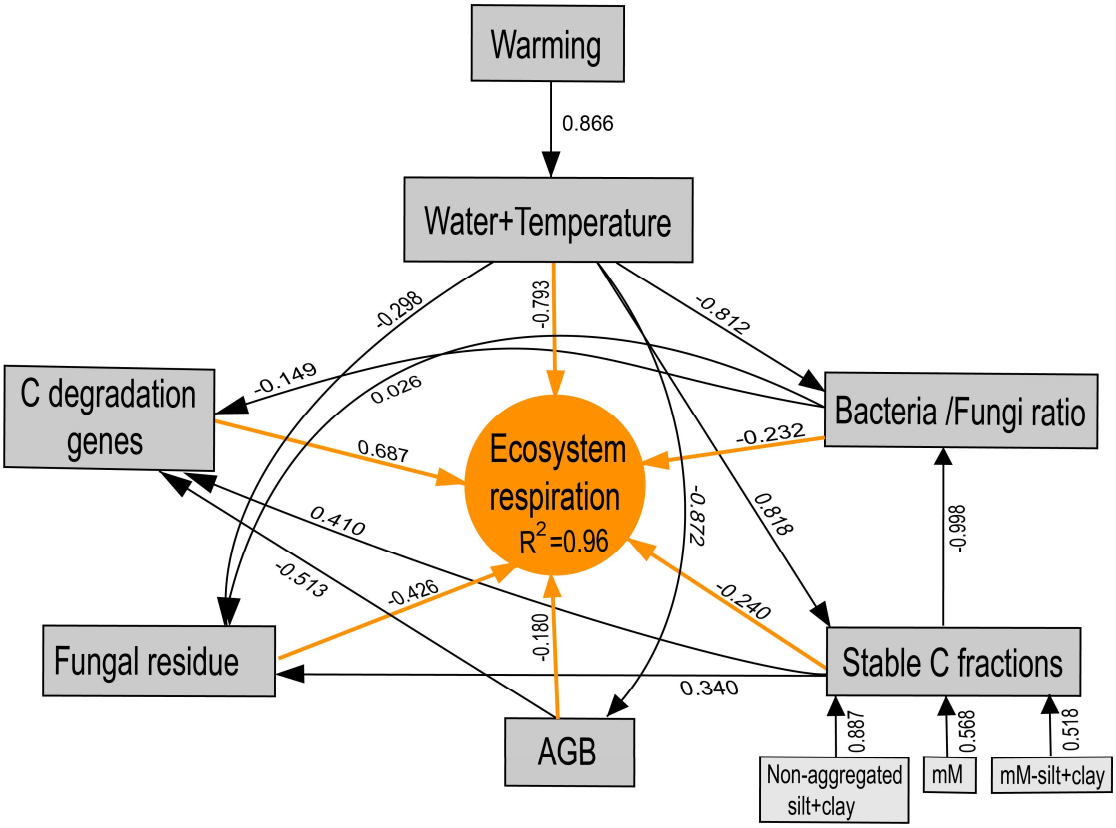

Figure 7 Partial least squares path analysis for ecosystem respiration, showing the relationship between selected biogeochemical processes, microbial community composition, and functional gene abundance

The orange arrows are the direct effect of environmental and microbial variables on ecosystem respiration, and the black arrows indicate the indirect path. The numbers listed within arrows are the standardized path coefficient. AGB: aboveground biomass; mM: microaggregates within macroaggregates.

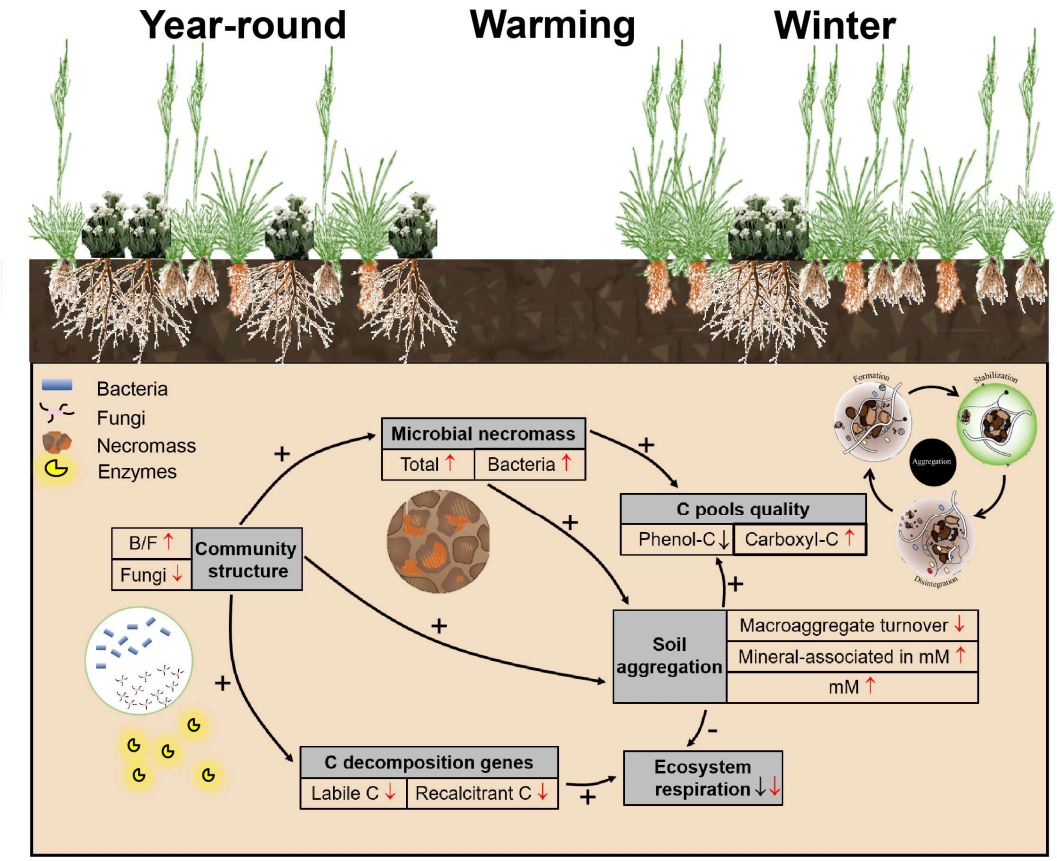

Figure 8 A conceptual diagrams illustrating the increasing or decreasing impact of warming on aggregate turnover, microbial community, necromass and the activity of microbial functional genes that control carbon cycling after year-round or winter warming as compared to control

The red and black arrows indicate the significant trends in winter and year-round warmed soil as compared to control. The (+) indicate the positive interactions between aggregate life cycle, microbial community, necromass and the potential activity of microbial functional carbon genes in winter warming condition.

Table 1 Effects of warming treatments on the proportions and soil organic carbon content of soil aggregate size fractions

Soil aggregate size fraction	CK		YW		WW	
	Size distribution (%)	C content (g C kg^{-1} soil)	Size distribution (%)	C content (g C kg^{-1} soil)	Size distribution (%)	C content (g C kg^{-1} soil)
Macroaggregates (M)	46.3 a ± 2.56	7.45 A ± 0.63	41.7 a ± 2.47	6.36 A ± 0.34	45.2 a ± 4.51	6.65 A ± 0.76
Free microaggregates (Fm)	40.2 a ± 1.70	6.07 A ± 0.28	37.4 a ± 0.50	4.44 C ± 0.07	38.9 a ± 2.41	5.26 B ± 0.31
Silt+clay	12.1 b ± 0.60	3.22 B ± 0.14	18.5 a ± 0.95	4.62 A ± 0.17	17.6 a ± 0.39	5.10 A ± 0.14
Coarse iPOM	10.2 a ± 0.89	0.66 A ± 0.11	8.30 a ± 0.68	0.60 A ± 0.07	9.10 a ± 0.67	0.67 A ± 0.07
mM	18.1 ab ± 0.84	2.49 AB ± 0.13	15.9 b ± 1.39	2.00 B ± 0.24	22.9 a ± 2.45	3.13 A ± 0.26
M-silt+clay	16.1 ab ± 1.45	3.61 A ± 0.37	12.1 b ± 1.55	3.49 A ± 0.11	16.6 a ± 0.85	3.14 A ± 0.34
Fine iPOM	0.29 a ± 0.05	0.36 AB ± 0.07	0.14 b ± 0.01	0.24 B ± 0.03	0.30 a ± 0.08	0.42 A ± 0.14
mM-POM	3.28 a ± 0.22	0.26 A ± 0.04	2.79 a ± 0.15	0.29 A ± 0.03	3.08 a ± 0.34	0.33 A ± 0.03
mM-silt+clay	14.8 ab ± 0.81	1.86 AB ± 0.15	13.4 b ± 1.50	1.50 B ± 0.20	19.9 a ± 2.23	2.37 A ± 0.29

Values are means ± standard error of means (n = 4).

Lowercase letters indicate significant differences in size distribution between treatments. Uppercase letters indicate significant differences in SOC contents between treatments. Abbreviations: CK: control; YW: year-round warming; WW: winter warming.

Coarse iPOM: coarse intra-aggregate particulate organic matter (inside macroaggregates but outside microaggregates); mM: microaggregates within macroaggregates; M-silt+clay: silt and clay-sized fractions inside macroaggregates; mM-POM: POM inside microaggregates within macroaggregates; mM-silt+clay: silt and clay-sized fractions inside mM; fine iPOM: fine intra-aggregate particulate organic matter (inside macroaggregates but outside microaggregates).