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Comparing microbial carbon sequestration and priming in the subsoil versus topsoil of a Qinghai-Tibetan alpine grassland

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ABSTRACT

Subsoils of alpine grasslands on the Qinghai-Tibetan Plateau represent a tremendous yet poorly investigated reservoir of soil organic carbon (SOC) on a global "hotspot" of warming. Compared with the temperature sensitivity of SOC decomposition, microbial anabolism of new carbon and priming of native SOC remain poorly constrained under warming-enhanced labile carbon input in these subsoils. Here we employed an innovative approach to investigate the sequestration of freshly added carbon in microbial necromass versus SOC priming in the top- (0–10 cm) and subsoils (30–40 cm) from a field experiment that simulated varied warming scenarios in an alpine grassland on the Qinghai-Tibetan Plateau. The ^{13}C composition of microbial necromass-derived amino sugars was analyzed in tandem with respired CO_2 and dissolved SOC components (including dissolved lignin) in an 86-day laboratory incubation with ^{13}C-labeled glucose. A higher fraction of freshly added carbon was respired while a smaller proportion was stabilized as amino sugars in the subsoil relative to the topsoil, leading to a much lower microbial carbon accumulation efficiency at depth. Meanwhile, a higher relative priming effect was observed in the subsoil (47± 14%) compared to the topsoil (14± 4%), suggesting a higher vulnerability to substrate-induced SOC loss at depth, although such changes may be associated with higher glucose addition rate (relative to SOC) in the subsoil. Furthermore, enhanced winter warming significantly reduced degradable SOC (assessed by SOC mineralization and dissolved lignin content) in the subsoil and potentially intensified nitrogen limitation under labile carbon additions, which further decreased microbial carbon accumulation (in the form of amino sugars) in the subsoil without affecting the topsoil. These results collectively indicate a limited microbial carbon sequestration potential and a higher vulnerability to warming-induced substrate changes in the subsoil of this alpine grassland, which warrants better understanding to predict soil carbon responses to climate warming on the Qinghai-Tibetan Plateau.

1. Introduction

The Qinghai-Tibetan Plateau is the highest plateau in the world, comprising an area of more than 2.4 million km^2 with an average altitude exceeding 4000 m a.s.l. (above sea level). More than 60% of the plateau's surface is covered by alpine grasslands (including meadows and steppe), whose soils store 33.5 Pg organic carbon (OC) in the top 0–75 cm, equivalent to 23.4% of soil organic carbon (SOC) stock in China (Wang et al., 2002). In the past 50 years, the plateau has experienced rapid climate warming with an average temperature increase of 0.2 °C per decade, especially in winter (Chen et al., 2013). This warming trend has further intensified since 2000 (Yao et al., 2007). With prolonged growing season and enhanced plant growth (Shen et al., 2015), warming is reported to induce a significant increase in net primary production (NPP) on the Qinghai-Tibetan Plateau (Wang et al., 2012), increasing plant OC input into soils. Meanwhile, SOC stock in the top 30 cm of alpine grasslands on the plateau has not changed from 1980 to 2004 (Yang et al., 2009), suggesting that increased carbon input may be counteracted by accelerated decomposition (Davidson and Janssens, 2006). Yet it remains to be investigated what

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mechanisms inhibit the build-up of SOC with increased NPP and whether SOC composition (or stability) has shifted under enhanced soil carbon cycling in order to better predict soil carbon response to climate warming on the Qinghai-Tibetan Plateau.

Climate warming may alter SOC cycling via at least two mechanisms: (i) enhancing microbial decomposition of soil organic matter (SOM) through accelerating enzymatic reaction at higher temperatures (Davidson and Janssens, 2006), and/or (ii) inducing "priming effect" (PE) on SOC through increased labile carbon supply to soil microbes (Fontaine et al., 2004; Hopkins et al., 2014) under enhanced plant growth or belowground allocations with warming (Xu et al., 2014; Walker et al., 2016). While the former has been extensively studied and recognized in soils (Allison and Treseder, 2008), SOC dynamics altered by warming-induced PE remain relatively poorly understood (Reinsch et al., 2013). As NPP increases with warming, plant inputs into the soil increase in the form of litter and root exudates, including carbohydrates, organic acids, and amino acids (Fontaine et al., 2004; Wild et al., 2014). These energy-rich and readily degradable compounds rapidly fuel catabolic and anabolic activities of soil microbes (Gunina and Kuzyakov, 2015), leading to potential shifts in microbial community composition (Hopkins et al., 2014) and increased (positive PE) or decreased (negative PE) decomposition of native SOC (Blagodatskaya and Kuzyakov, 2008; Kuzyakov, 2010).

Mechanisms governing the direction of PE are still unclear (Georgiou et al., 2015) but nutrient availability has been suggested to play a decisive role. Under high nutrient availabilities, microbes switch from SOM decomposition to labile OC utilization, leading to negative priming ("preferential substrate utilization" hypothesis; Blagodatskaya et al., 2007). On the opposite, under low nutrient availabilities soil microbes utilize labile OC to synthesize extracellular enzymes for the acquisition of nutrients from SOM, thereby leading to positive priming ("microbial nutrient mining" hypothesis; Craine et al., 2007). Given that nitrogen (N) mineralization rates are restricted by low temperatures in alpine grasslands, plants and microbes may compete strongly for inorganic N (Xu et al., 2006). It is hence worth investigating whether warming induces positive priming of native SOC on the Qinghai-Tibetan Plateau and how it affects SOC quality as well as quantity. Furthermore, subsoils (>30 cm) in the first meter store more than 47% of SOC on the Qinghai-Tibetan Plateau (Yang et al., 2009) and have distinct abiotic (e.g., soil texture, reactive minerals and permafrost distribution) and biotic (e.g., microbial biomass and diversity) properties from the topsoil (Ollivier et al., 2013). The response of subsoil OC to warming and labile carbon addition can be different from that of topsoil (Fierer et al., 2003a; Wild et al., 2014) and will be pivotal in predicting the change of SOC storage under climate warming.

In contrast to priming, microbial anabolism may increase SOC content in the form of microbial residues (mainly necromass) and represents a not-yet-fully-investigated fate of fresh carbon added into soils (Liang et al., 2011). Microbial cell wall components such as amino sugars are an important part of microbial residue or necromass (Engelking et al., 2007; He et al., 2011). The most important amino sugars in soils include glucosamine mainly derived from fungal cell walls, galactosamine, and muramic acid exclusive to bacteria (Zhang and Amelung, 1996). These compounds are relatively stable in soils and are hence used as a time-integrated measure of microbial carbon not subject to rapid fluctuations (Liang and Balser, 2012). Due to the slow turnover of microbial necromass (Liang et al., 2011), it is difficult to investigate amino sugar dynamics in the short term. However, coupled with intensive isotopic labeling, these compounds may be utilized to probe microbial sequestration of newly added carbon in soils (He et al., 2011), which may counteract positive priming of SOC.

Here we conduct a ^{13}C-labeled priming experiment on the topsoil (0–10 cm) and subsoil (30–40 cm) from a field warming study on an alpine grassland of Qinghai-Tibetan Plateau. Our approach serves two purposes. First, with the amendment of ^{13}C-labeled glucose, we attempt to simulate labile carbon increase resulting from warming-induced plant growth and to assess the response of microbial anabolism to freshly added carbon (assessed by novel δ^{13}C measurement of individual amino sugars) and degradation of native SOC (via respiration in the form of CO_2). Second, we examine SOC mineralization in the priming experiment to evaluate changes in the degradability or quality of SOM collected from different field warming treatments. We hypothesize that labile carbon input stimulates both microbial carbon accumulation and native SOC decomposition, which in turn diminishes easily degradable substrates in the soil and decreases SOC degradability (or quality) under warming. These effects may be particularly pronounced in the subsoil that is relatively depleted of energy-rich OC and are hence worthy of comparison with the topsoil in terms of predicting SOC responses to warming.

2. Materials and methods

2.1. Study site and soil sampling

The field warming experiment is located at the Haibei Alpine Grassland Ecosystem Research Station (101°19′E, 37°36′N, mean elevation of 3215 m a.s.l.), which lies on the northeastern Qinghai-Tibetan Plateau. The region has a continental monsoon climate with a mean annual temperature of −1.2 °C and a mean annual precipitation of 489 mm, 80% of which occurs during the summer monsoon season. Soils at the site are Mat-Gryic Cambisol with a relatively high SOC content (~7% at 0–10 cm), a clay loam texture (Table 1) and a mean pH of 8.01 (measured in 2.5 ml of water per gram of soil). The native plant community is dominated by *Kobresia humilis, Elymus nutans, Stipa aliena* and *Gentiana straminea*.

A multi-factorial field experiment was established at the Haibei Research Station with the aim to investigate effects of warming and altered precipitation on plant communities and soil processes (Fig. 1). The manipulative experiment was a full factorial design, initially including two warming levels [control without warming (CK) and regular whole-year warming (W1)] and three precipitation levels (drought, ambient and wet) in July 2011. Each treatment had six independent and randomly distributed replicates (1.8 m × 2.2 m per plot) on a relatively homogeneous landscape in terms of geomorphology and soil properties. The experiment was expanded subsequently to include an enhanced winter warming (W2) treatment with 5 replicates in January 2012. For this study we focused on CK, W1 and W2 treatments to examine warming effects on the degradability of native SOC.

The W1 and W2 plots were heated by infrared heaters installed 1.6 m above the soil surface while dummy heaters were installed above the CK plots. Heating started immediately after installation and surface soil temperature was monitored continuously at depths of 5 cm, 10 cm and 20 cm. Compared with the CK plots, surface soil temperature increased by an average of 2 °C throughout the year in the W1 plots, and increased by ~3 °C in winter (mid-October to mid-April) and by 0.5~1 °C for the rest of the year in the W2 plots. On an annual average basis, W1 and W2 treatments represented similar degree of warming but varied scenarios of seasonality. As the Tibetan Plateau is experiencing stronger warming trend in the winter (Chen et al., 2013), W2 treatment represents a more likely seasonality of future warming and likely has a stronger influence on the freeze-thaw cycles or seasonal permafrost dynamics in the soil.

In August 2013, after ~2 years of W1 treatment, we selected three plots in each of the CK, W1 and W2 treatments and collected three soil cores (diameter of 3 cm) up to the depth of 70 cm from

Table 1
Soil bulk properties in the top- and subsoils of Haibei Research Station warming experiment (mean ± standard error; n = 3). CK: control treatment, W1: whole-year warming, W2: enhanced winter warming, SOC: soil organic carbon, N: nitrogen, MBC: microbial biomass carbon, MBN: microbial biomass nitrogen.

	Topsoil (0–10 cm)			Subsoil (30–40 cm)		
	CK	W1	W2	CK	W1	W2
SOC (mg C g^{-1} soil)	70.9 ± 0.8	75.4 ± 6.4	75.7 ± 3.8	17.6 ± 2.3	20.7 ± 2.9	19.2 ± 0.9
N (mg N g^{-1} soil)	5.7 ± 0.4	5.2 ± 1.0	5.6 ± 0.3	1.7 ± 0.2	1.4 ± 0.3	1.6 ± 0.1
SOC:N	12.6 ± 1.0	15.4 ± 2.4	13.7 ± 1.3	10.4 ± 0.9	15.7 ± 3.1	11.8 ± 0.7
MBC (μg C g^{-1} soil)	627 ± 36	655 ± 25	641 ± 37	135 ± 28	127 ± 38	129 ± 8
MBN (μg N g^{-1} soil)	61.2 ± 2.7	60.3 ± 7.3	64.8 ± 1.2	12.7 ± 2.8	12.0 ± 1.7	10.9 ± 1.6
MBC:SOC (%)	0.88 ± 0.05	0.88 ± 0.06	0.85 ± 0.02	0.79 ± 0.17	0.68 ± 0.26	0.67 ± 0.05
Clay (%)	58.4 ± 2.8	60.0 ± 3.3	54.4 ± 3.0	71.8 ± 0.5	68.9 ± 3.4	69.3 ± 1.7
Silt (%)	6.2 ± 0.5	8.1 ± 1.5	6.0 ± 0.8	21.7 ± 1.0	19.9 ± 1.3	18.6 ± 0.2
Sand (%)	35.5 ± 3.2	31.9 ± 4.8	41.4 ± 1.6	6.5 ± 0.8	11.2 ± 4.3	13.9 ± 1.9

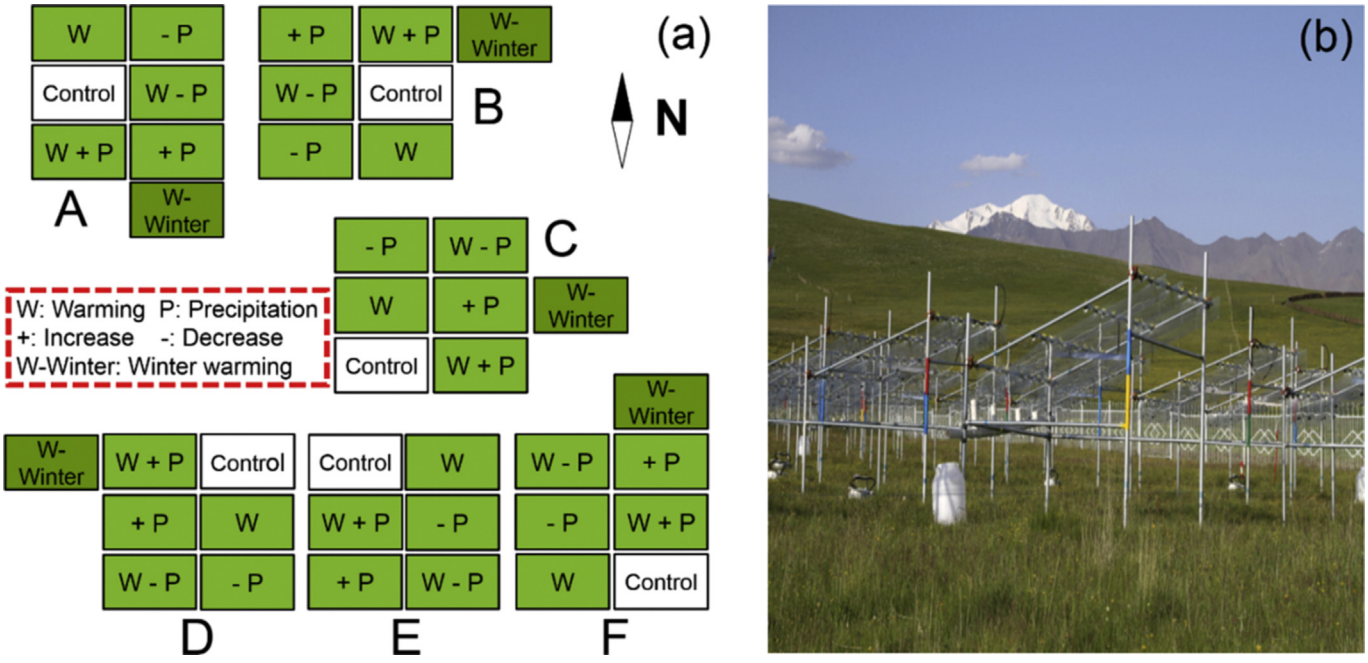

Fig. 1. Warming and precipitation control experiment at the Haibei Research Station: (a) layout of the experimental design; (b) photographs of the experiment platform. The CK, W1 and W2 treatments in this paper correspond to the Control, W and W-Winter in (a), respectively.

each plot. Soils from the same plot and same depth (0–10, 10–20, 20–30, 30–40 and 40–70 cm, respectively) were homogenized. Visible roots were hand-picked before the soil was passed through a 2-mm sieve. Soils were then stored in a refrigerator at 4 °C (<10 days) until further analyses or experiment. Based on the preliminary results, warming increased belowground NPP in the depth of 30–50 cm (He et al., unpublished data), presumably increasing labile carbon input to this layer. Hence, both the topsoil (0–10 cm) and subsoil (30–40 cm) were used for this study with an average bulk density of 0.82 and 1.27 g m^{-3}, respectively.

2.2. Soil bulk properties

SOC content was measured by the potassium dichromate oxidation-ferrous sulfate titrimetry method (Nelson et al., 1996) while total N content was determined as ammonium-N by steam distillation after digestion with H_2SO_4 (Kjeldahl method; Bremner et al., 1996). Soil clay, silt and sand contents were analyzed by laser diffraction using Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). Microbial biomass carbon (MBC) and nitrogen (MBN) were determined for field-moist soils upon sampling using chloroform fumigation-extraction method (Vance et al., 1987) with modifications (Wu et al., 1990). MBC or MBN were calculated as dissolved OC (DOC) or N differences between fumigated and non-fumigated samples divided by a factor of 0.45 for DOC (Joergensen, 1996) and 0.54 for N (Joergensen and Mueller, 1996), with DOC and N analyzed on a Multi N/C 3100-TOC/TN Analyzer (Analytik Jena, Germany).

2.3. Priming experiment and CO_2 analysis

An 86-day incubation was conducted in the laboratory at 25 °C in the dark on the top- and subsoils selected from the CK, W1 and W2 treatments to investigate SOC mineralization and microbial carbon dynamics with or without glucose amendment (n = 3). For both incubations, ~10 g (dry weight) of sieved moist soil were placed in a 165-ml brown glass flask and pre-incubated at 25 °C in the dark for one week. Subsequently, water solutions with ^{13}C-labeled glucose (Sigma-Aldrich, uniformly labeled, 99 atom%) or distilled water were sprayed onto the soil to reach a moisture content equivalent to 65% of its water holding capacity (WHC). Soil moisture content was maintained by weighing and spraying MilliQ water regularly. To examine the maximum potential of SOC mineralization, we used a glucose addition rate of 1.4 mg C g^{-1} soil

based on the annual belowground NPP in the topsoil at this site (300–400 g m^{-2} for 0–10 cm; He et al., unpublished data; assuming a soil bulk density of 1.0 g cm^{-3} and an OC content of 50% for roots). The amount of added glucose corresponds to 2% and 8% of SOC or 2- and 12-fold MBC in the top- and subsoils, respectively, well beyond the linear increase range of PE in response to substrate increases (<50% MBC) summarized by Blagodatskaya and Kuzyakov (2008), which includes activated microbial degradation of SOC (real priming) and accelerated turnover of microbial biomass (apparent priming). With this high dose of labile carbon addition, we also hope to fully alleviate carbon limitation to soil microbes in an attempt to examine warming-induced changes in SOC degradability and to trace freshly added carbon incorporated into microbial residues in a relatively short period of time. Potential influences on the experiment results caused by the large amount of added OC are discussed later.

Soil respiration was measured on Days 1, 3, 7, 11, 15, 20, 50, 76 and 86 by quantifying CO_2 on a gas chromatograph (GC; Agilent 7890A, USA) coupled with flame ionization detector (FID). The incubation was stopped on Day 86 when soil respiration rates were similar in the respective samples with and without glucose amendment, indicating the end of PE. To differentiate SOM- and glucose-derived CO_2, the $\delta^{13}C$ value of respired CO_2 was measured periodically (5 times in total) on an isotope ratio mass spectrometry (IRMS; GasBench II, Delta PLUS Advantage, Thermo Finnigan, Bremen, Germany) after dilution with a standard CO_2 gas of known concentration and isotopic composition. The ^{13}C abundance of respired CO_2 was corrected for the added CO_2 gas using a mass balance approach.

For samples with glucose amendment, the contribution of SOM-derived OC to the respired CO_2 was calculated by the mass balance equations:

$$R_S + R_G = R_T \tag{1}$$

$$R_S \times A_S^{13} + R_G \times A_G^{13} = R_T \times A_T^{13} \tag{2}$$

where R_T, R_S and R_G are the cumulative CO_2 (mg C g^{-1} soil) respired from the total sample, derived from SOM, and from glucose, respectively (c.f. Table S1). *A13 T*, *A13 S* and *A13 G* are the ^{13}C abundance (dimensionless) of respired CO_2, SOM (1.08 atom%) and added glucose (99 atom%), respectively.

PE induced by glucose amendment (mg C g^{-1} soil) and its relative percentage compared with soil respiration without glucose amendment (relative PE) was calculated as:

$$PE = R_S - R_{No\ glucose} \tag{3}$$

$$Relative\ PE = PE \Big/ R_{No\ glucose} \times 100\% \tag{4}$$

where $R_{No\ glucose}$ is the cumulative CO_2 respired from soil without glucose amendment.

2.4. Water-extractable OC (WEOC) and dissolved lignin analysis

To determine the "labile" fraction of SOC, WEOC was extracted by mixing air-dried soils (5–8 g) with 35 ml of MilliQ water on a reciprocal shaker for 10 h. The supernatant was filtered through pre-combusted 0.7-μm glass microfiber filters (GF/F, Whatman) to lower background contamination. A small aliquot was analyzed on a Multi N/C 3100-TOC/TN Analyzer (Analytik Jena, Germany) for WEOC content after acidifying to pH 2 with concentrated HCl.

Dissolved lignin was isolated on the same day from the remaining water extracts using modified CuO oxidation method (Hedges and Ertel, 1982; Hernes and Benner, 2002). Briefly, water extracts (20 ml) were mixed with 50 mg CuO, 10 mg ammonium iron (II) sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) and 10 mg of glucose in N_2-purged NaOH solution (final concentration of 2 M) in Teflon-lined bombs and heated at 150 °C for 150 min. The oxidation products were recovered with ethyl acetate at pH 2, spiked with ethyl vanillin (recovery standard) and *trans*-cinnamic acid (internal standard) and quantified as trimethylsilyl (TMS) derivatives on a gas chromatography-mass spectrometry (GC-MS; Thermo Fisher Scientific, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The concentration of dissolved lignin was reported as the summation of eight characteristic lignin phenols, including vanillyls (vanillin, acetovanillone and vanillic acid), syringyls (syringaldehyde, acetosyringone and syringic acid), and cinnamyls (*p*-coumaric acid and ferulic acid).

2.5. Quantification and ^{13}C isotopic analysis of amino sugars

Amino sugars were extracted from air-dried incubated soils (0.06–0.3 g; ground to < 0.25 mm) according to Zhang and Amelung (1996). Myo-inositol and methyl-glucamine were added as internal and recovery standards, respectively. Aliquots of the amino sugars were converted into aldononitrile derivatives and quantified on a GC-FID (Agilent 6890A, USA) equipped with a HP-5 capillary column (30 m × 0.25 mm × 0.25 μm). The ratio of glucosamine to muramic acid was used to indicate the relative contribution of fungi versus bacteria to soil microbial residual carbon (Joergensen and Wichern, 2008).

The ^{13}C composition of two major amino sugars with sufficient abundance in the soil (glucosamine and galactosamine) was determined on a GC-combustion-isotope ratio MS (GC-C-IRMS; Delta plus, Thermo Finnigan MAT 253, Germany) equipped with a DB-5MS capillary column (60 m × 0.25 mm × 0.25 μm) after derivatization with acetic anhydride following Glaser and Gross (2005). The $\delta^{13}C$ values of soil amino sugars were calculated according to the equation:

$$No._{Amino\ sugar} \times \delta^{13}C_{Amino\ sugar} + No._{Acet} \times \delta^{13}C_{Acet} = No._{Der} \times \delta^{13}C_{Der} \tag{5}$$

where No. is the number of C atoms in the amino sugar molecule ($No._{Amino\ sugar} = 6$), the acetyl group used for derivatization ($No._{Acet} = 10$) and the amino sugar derivatives ($No._{Der} = 16$). The $\delta^{13}C$ of derivative carbon ($\delta^{13}C_{Acet}$) was estimated separately using amino sugar standards (Glaser and Gross, 2005).

Similar to the CO_2 analysis, we used a mass balance approach to calculate the contribution of glucose-derived carbon to individual amino sugars at the end of the incubation:

$$f_G + f_S = 1 \tag{6}$$

$$f_G = \left(\delta^{13}C_T - \delta^{13}C_S\right) \Big/ \left(\delta^{13}C_G - \delta^{13}C_S\right) \tag{7}$$

where f_G and f_S are the percentage of amino sugars derived from ^{13}C-labeled glucose and SOC, respectively (Table S1). $\delta^{13}C_G$, $\delta^{13}C_T$ and $\delta^{13}C_S$ are the $\delta^{13}C$ values of glucose and individual amino sugars measured in glucose-amended and non-amended soils, respectively. Amino sugars derived from glucose were synthesized during incubation whereas those derived from SOC included contribution of microbial necromass accumulated both before and during incubation.

To compare microbial accumulation of freshly added carbon, we calculated microbial carbon accumulation efficiency (CAE) defined

as:

$$CAE = AS_G/(AS_G + R_G) \quad (8)$$

where AS_G is the concentration of amino sugars synthesized using glucose (μg C g^{-1} soil), calculated by multiplying f_G with amino sugar concentration (converted to a carbon content of 40%) in the respective soil after incubation. The CAE calculated here most likely underestimates microbial carbon accumulation in the soils as amino sugars represent only part of the microbial necromass synthesized from glucose during incubation. Nevertheless, it provides a benchmark to assess potential shifts in microbial CAE induced by warming. CAE is different from microbial carbon use efficiency (CUE) in two ways. First, CAE is much smaller in value than CUE because only a small fraction of microbial biomass is preserved as necromass in the soil. Second, CAE takes into account the preservation efficiency of microbial biomass/necromass besides growth efficiency. Hence, CAE should provide new insights into microbial carbon sequestration in soils.

2.6. Statistical analyses

All statistical analyses were performed using SPSS 18.0 (SPSS, Chicago, USA). We assessed homogeneity of variances and normal distribution of the data using Shapiro-Wilk test before applying parametric methods and performed log transformation where necessary. Non-parametric tests (Kruskal-Wallis or Wilcoxon) were conducted if normal distribution was not achieved. Differences in the R_S:R_G ratio, relative PE and CAE between warming treatments and soil depths were assessed by two-way ANOVA (General Linear Model in SPSS) with warming treatment and soil depth as fixed effects. No significant interaction effect between warming treatment and soil depth was observed. One-way ANOVA was hence used to compare differences among warming treatments at the same depth where necessary. *T* test was used to examine differences between depths of the same warming treatment and the effect of glucose addition on Rs, amino sugar content and ratio of glucosamine:muramic acid. Differences are considered to be significant at a level of $p < 0.05$.

3. Results

3.1. Soil bulk properties and MBC contents

Soil bulk properties are listed in Table 1. SOC ranged from 70.9 ± 0.8 to 75.7 ± 3.8 mg C g^{-1} soil (mean ± standard error) in the topsoil and from 17.6 ± 2.3 to 20.7 ± 2.9 mg C g^{-1} soil in the subsoil. N contents varied around 5–6 mg N g^{-1} soil in the topsoil and decreased to 1–2 mg N g^{-1} soil in the subsoil. The resulting SOC:N ratio (10–16) did not vary significantly between depths. Soil MBC contents decreased from >600 to ~130 μg g^{-1} soil with depth (n = 9; $p < 0.05$), accounting for 0.85–0.88% and 0.67–0.79% of SOC in the top- and subsoils, respectively. MBN was also higher (>60 μg g^{-1} soil) in the topsoil than in the subsoil (<13 μg g^{-1} soil; $p < 0.05$). In contrast, clay and silt contents were higher in the subsoil (n = 9; $p < 0.05$). None of these soil properties differ significantly among varied warming treatments at either depth at the time of sampling.

3.2. CO_2 efflux and priming induced by glucose amendment

A total of 3.6 ± 0.3 and 1.0 ± 0.1 mg C g^{-1} was respired from the non-amended top- and subsoils of Haibei alpine grassland during incubation, respectively (i.e., $R_{No\ glucose}$; Figs. 2 and S1). $R_{No\ glucose}$ had a similar proportion relative to the initial SOC content at both depths (~5%) and did not vary significantly among different warming treatments. In glucose-amended soils, R_S accounted for >67% of R_T (i.e., R_G + R_S) at both depths and was higher in the topsoil than in the subsoil ($p < 0.05$; Fig. 2). R_S did not differ among warming treatments in the topsoil but was significantly higher in the subsoil of the CK relative to the W2 treatment ($p < 0.05$; Fig. 2). R_G was significantly higher in the subsoil than in the topsoil ($p < 0.05$), leading to higher R_S:R_G ratios in the topsoil across all warming treatments ($p < 0.05$; Fig. 3a). Due to high variability of the data, no significant difference of R_T, R_G was found among varied warming treatments for either depth.

R_S was significantly higher than $R_{No\ glucose}$ in soils of the CK treatment ($p < 0.05$; Fig. 2), confirming positive priming of SOC by glucose amendment in these soils. PE was 0.52 ± 0.13 and 0.40 ± 0.11 mg C g^{-1} soil, corresponding to 0.73± 0.18% and 2.33± 0.72% SOC in the top- and subsoils (n = 9), respectively. To further compare priming-induced SOC loss with $R_{No\ glucose}$, we calculated relative PE (c.f. Table S1). Relative PE was higher in the subsoil (46± 14%) than in the topsoil (14± 4%; n = 9; $p < 0.05$) but did not show any significant difference among varied warming treatments at either depth (Fig. 3b).

3.3. WEOC and dissolved lignin content

WEOC content was higher in the topsoil (0.24 ± 0.03 mg C g^{-1} soil) than in the subsoil (0.09 ± 0.01 mg C g^{-1} soil; n = 9; $p < 0.05$) before incubation. WEOC did not change after incubation without glucose amendment and did not differ among varied warming treatments (Fig. 4a). Dissolved lignin content was also higher in the topsoil (24.5 ± 3.4 μg C g^{-1} SOC) than in the subsoil (14.9 ± 2.2 μg C g^{-1} SOC; n = 9; $p < 0.05$) before incubation and was similar in the topsoil before and after incubation (Fig. 4b). By comparison, dissolved lignin was lower in the subsoil of W2 than the CK and W1 treatments before incubation ($p < 0.05$). It decreased or remained similar in the subsoil of the CK and W1 treatments after incubation but increased significantly in the glucose-amended subsoil of W2 ($p < 0.05$; Fig. 4b).

3.4. Amino sugar content and composition

Amino sugars were quantified in all soils at the end of the

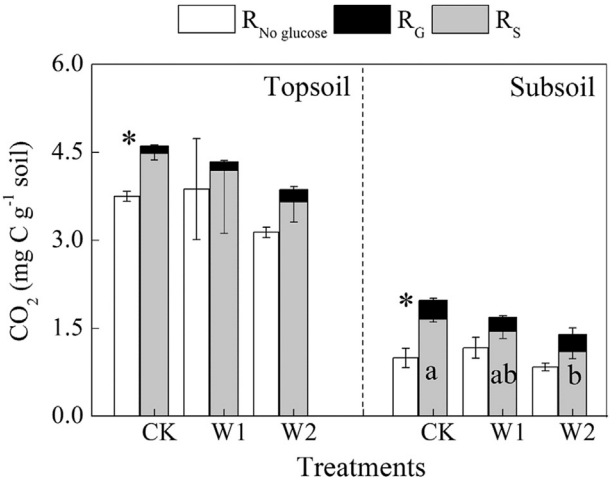

Fig. 2. Cumulative CO_2 effluxes in the 86-day incubation experiment. R_G, R_S and $R_{No\ glucose}$ are defined in Table S1. Mean values are shown with standard error (n = 3). * indicates significant difference between R_S and $R_{No\ glucose}$ (*t*-test; $p < 0.05$). Different letters indicate significant difference among the control (CK), whole-year warming (W1) and winter-enhanced warming (W2) treatments at the same soil depth (one-way ANOVA; $p < 0.05$).

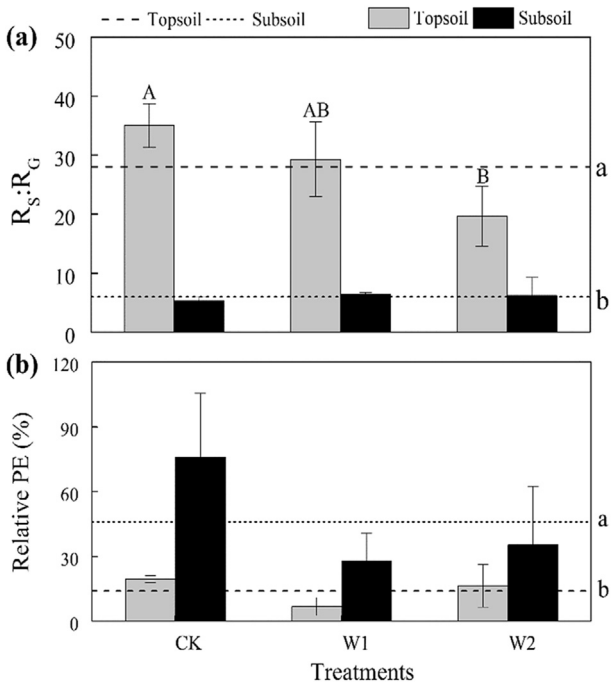

Fig. 3. Ratios of R_S:R_G (a) and relative PE (b) in the top- and subsoils of Haibei alpine grassland after incubation. Abbreviations are defined in Table S1 and Fig. 2. Mean values are shown with standard error (n = 3). Bold dash and dotted lines represent mean values for all the top- and subsoils (n = 9), respectively. Different letters indicate significant difference between top- and subsoils (two-way ANOVA; $p < 0.05$).

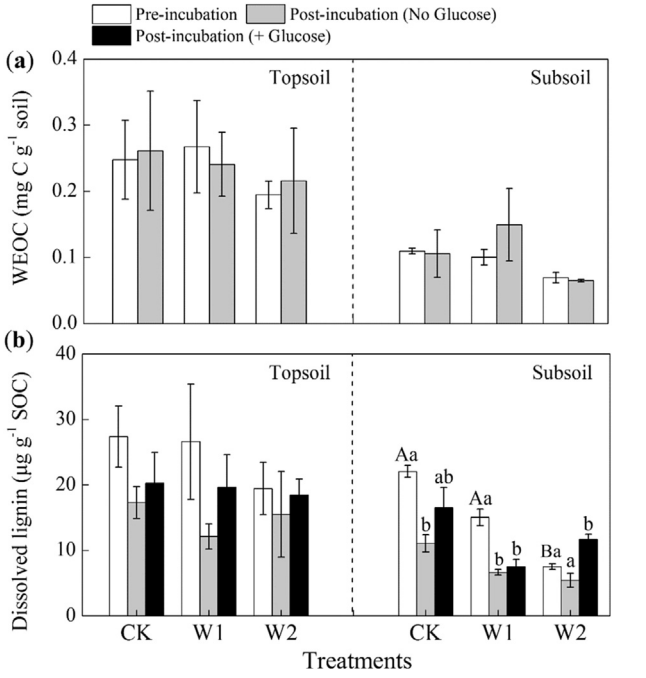

Fig. 4. Changes of water-extractable organic carbon (WEOC; a) and dissolved lignin (b) contents in the soil before and after incubation. Mean values are shown with standard error (n = 3). Different lowercase letters indicate significant difference among pre- and post-incubation samples of the same warming treatment while different uppercase letters indicate significant difference among varied warming treatments in pre-incubation soils (one-way ANOVA; $p < 0.05$).

incubation. Glucosamine had the highest abundance, accounting for 36–64% of three major amino sugars, followed by galactosamine and muramic acid (Fig. 5a–c). Total amino sugars varied around 3.2–5.8 mg g^{-1} soil in the topsoil and 0.7–1.4 mg g^{-1} soil in the subsoil without glucose amendment (Fig. 5d). Given the OC content of amino sugars (40%), amino sugar carbon was equivalent to ~2% SOC and ~3 times of MBC in all soils. By comparison, glucose-amended incubation decreased total amino sugar content by ~8% in the topsoil of all treatments and by ~24% in the subsoil of both warming treatments. As a result, total amino sugar content was significantly lower in the glucose-amended subsoil of the W2 treatment relative to no-glucose incubations ($p < 0.05$; Fig. 5d). Muramic acid was substantially lower in glucose-amended soils relative to no-glucose incubations ($p < 0.05$; Fig. 5c). Amino sugar content was not affected by varied warming treatments, except that glucosamine was significantly lower in the glucose-amended subsoil of W2 compared with CK ($p < 0.05$, Fig. 5a).

The glucosamine:muramic acid ratio was much higher (average of ~9) in the topsoil than in the subsoil (~2) without glucose amendment ($p < 0.05$, Fig. 6), indicating a higher contribution of fungi-derived residual carbon in the topsoil. Due to decreased concentration of muramic acid, the glucosamine:muramic acid ratio was significantly higher in glucose-amended soils ($p < 0.05$), especially in the subsoils (Fig. 6). The ratio did not show any difference among varied warming treatments.

3.5. Amino sugar $\delta^{13}C$ values and CAE

Glucosamine and galactosamine exhibited $\delta^{13}C$ values of −30 and −31‰ in the topsoil and −27 and −26‰ in the subsoil without glucose amendment (n = 3), respectively. Both amino sugars were substantially enriched with ^{13}C after glucose-amended incubation owing to the intensive ^{13}C labeling we used, even though only a tiny fraction of total amino sugars was synthesized from added glucose ($f_G < 0.03\%$; Table 2). These proportions were equivalent to a total AS_G concentration of 0.03–0.09 μg C g^{-1} in soils (including both glucosamine and galactosamine; Table 2), which was significantly lower in the subsoil than in the topsoil ($p < 0.05$). Compared with R_G, AS_G was very small fraction of amended glucose, representing <0.01% of the carbon amended, resulting in low CAE values (0.01–0.09%). In general, CAE was lower in the subsoil (0.02± 0.003%) than in the topsoil (0.04 ± 0.006%; n = 9; $p < 0.05$; Fig. 7). CAE was not affected by varied warming treatments at either depth.

4. Discussion

4.1. Higher vulnerability of alpine grassland subsoil to priming compared with the topsoil

The subsoil of Haibei alpine grassland had a much lower ratio of R_S:R_G than the topsoil in our experiment (Fig. 3a), suggesting a microbial catabolic preference for labile substrate in the deeper soil, likely resulting from the lower availability or abundance of labile SOC at depth (Fontaine et al., 2007; De Graaff et al., 2014). Admittedly, our SOC-normalized glucose amendment rate was higher in the subsoil (8% SOC) relative to the topsoil (2% SOC) due to a lower SOC content, which may affect R_G because glucose mineralization generally increases with increasing addition rate in the relatively low to median range (0.0009–288 μg C g^{-1} soil or < 1.8% of SOC; Schneckenberger et al., 2008). However, both Bremer and Kuikman (1994) and Schneckenberger et al. (2008) showed the existence of an upper limit of glucose amendment rate above which further additions do not affect glucose mineralization. Our study used a glucose amendment rate well above the upper limit (~0.3 mg C g^{-1} soil or 1.8% of SOC) tested by Bremer and Kuikman (1994).

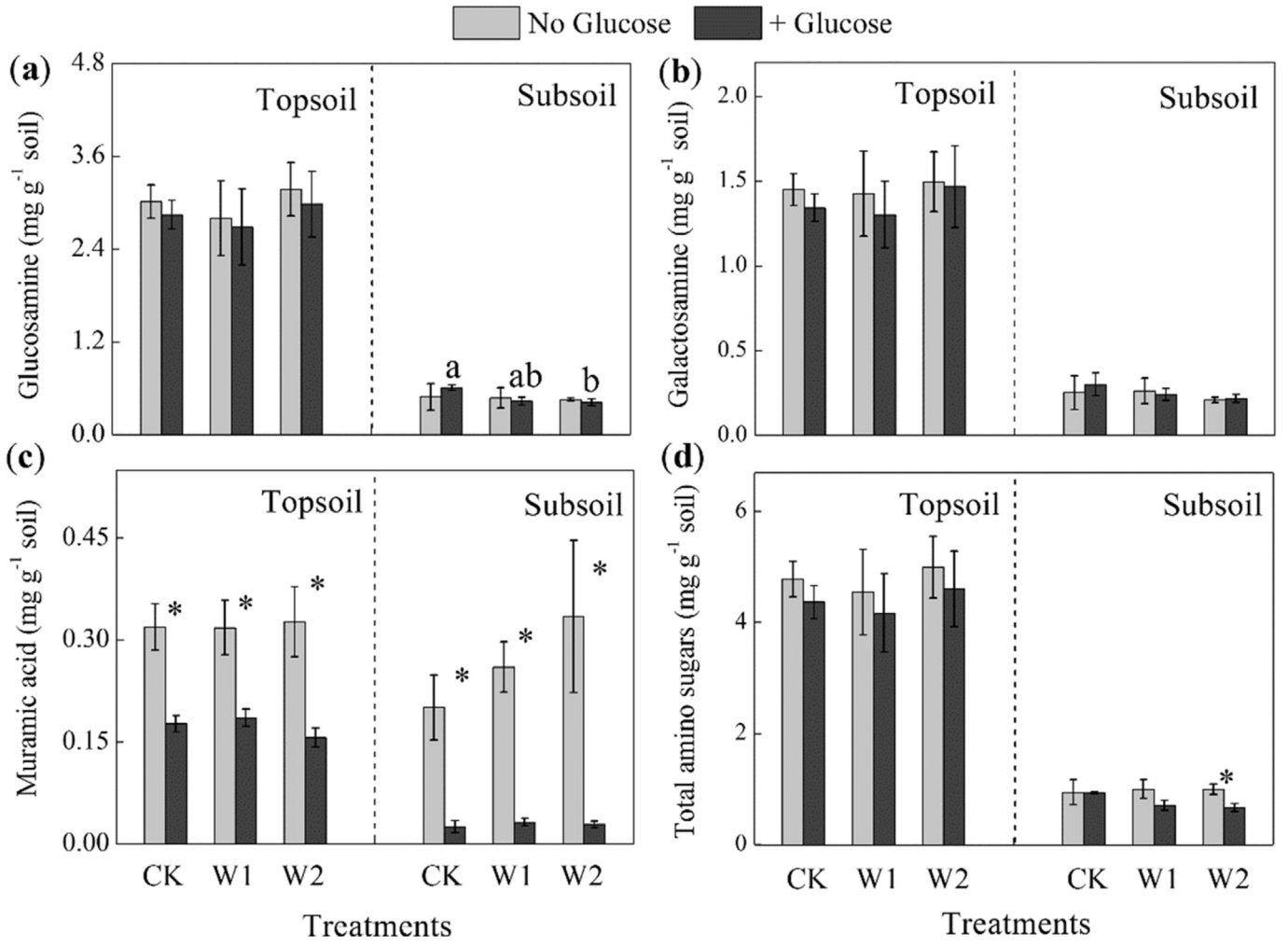

Fig. 5. Concentrations of individual and total amino sugars in the top- and subsoils after incubation with or without glucose. Mean values are shown with standard error (n = 3). * indicates significant difference between glucose- and non-amended soils of the same warming treatment (*t*-test; $p < 0.05$). Different letters indicate significant difference among varied warming treatments at the same soil depth (one-way ANOVA; $p < 0.05$).

Therefore, R_G was probably not affected by the higher SOC-normalized glucose addition rate in the subsoil.

Despite a labile substrate preference, the subsoil in our experiment showed a higher relative PE than the topsoil (Fig. 3b), suggesting a higher vulnerability to substrate-induced SOC loss at depth. Similar to R_G, we must consider the effect of glucose addition rate on PE in the top-versus subsoils (Blagodatskaya and Kuzyakov, 2008; Paterson and Sim, 2013; Wang et al., 2015). As Blagodatskaya and Kuzyakov (2008) reviewed, an exponential decrease of PE or even a switch to negative values is often observed when carbon amendment is higher than 50% of MBC. With higher labile carbon input (1.5–3.2 mg C g^{-1} soil or 14–30% of SOC), Guenet et al. (2010) reported that PE intensity varied comparatively little between different amendment levels and suggested that PE is a saturating function of labile carbon input. However, a positive dose-dependence of SOC priming has also been demonstrated in several studies (Paterson and Sim, 2013; Rousk et al., 2015). In our experiment, a higher carbon amendment rate was used in the subsoil (12-fold MBC or 8% SOC) compared to the topsoil (twice MBC or 2% SOC) and the subsoil had a similar PE or higher relative PE compared to the topsoil. Furthermore, the ratio of PE to glucose amendment rate was similar in top- and subsoil (0.4) rather than decreased with increasing amount of exogenous substrate as observed in other studies (Paterson and Sim, 2013; Wang et al., 2015). These results collectively indicate that the Haibei alpine grassland subsoil may be particularly sensitive to labile carbon additions. Our results are in line with elevated priming in grassland subsoil reported previously (Fierer et al., 2003a; Fontaine et al., 2007) and imply that the stability of subsoil SOC is mediated by the supply of labile carbon. Meanwhile, it must be re-emphasized that glucose amendment rate varied considerably relative to SOC in the subsoil versus topsoil. Potential bias induced by varied carbon addition intensity cannot be completely ruled out with our current data and merits further investigation in our future experiments.

The relative PE observed in our experiment is within the range reported for other soils using a similar carbon amendment rate (Hamer and Marschner, 2005; Guenet et al., 2010, 2012; Paterson and Sim, 2013; Wang et al., 2015). However, PE in the subsoil of our experiment corresponds to about 2.3% of SOC, higher than those reported elsewhere. For instance, PE accounted for 0.3–1.2% of SOC in arable soils with a carbon amendment rate of ~8% SOC (Guenet et al., 2012). With carbon addition rate equivalent to 9- and 13-fold MBC, only 0.6 and 0.2% of SOC were primed as CO_2 in forest soils, respectively (Hamer and Marschner, 2005; Wang et al., 2015). Our results imply that a higher fraction of SOC of the Haibei alpine grassland subsoil is prone to positive priming induced by substrate input increases, which warrants better understanding in warming-

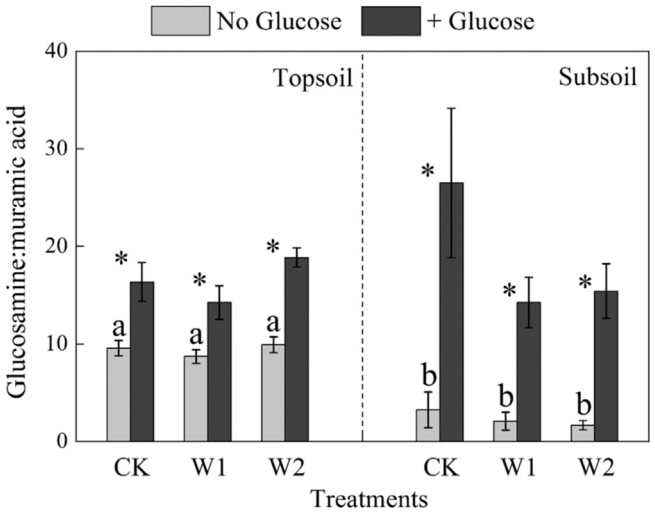

Fig. 6. Ratios of glucosamine:muramic acid in soils after incubation with or without glucose. Mean values are shown with standard error (n = 3). * indicates significant difference between glucose- and non-amended soils of the same treatment (*t*-test; $p < 0.05$). Different letters indicate significant difference between depths in non-amended soils (*t*-test; $p < 0.05$). Note that glucose-amended soils have similar ratios among all treatments and at both depths.

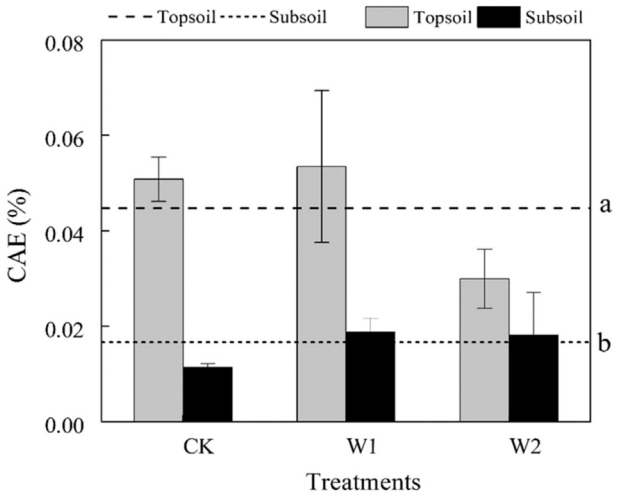

Fig. 7. Carbon accumulation efficiency (CAE) in the top- and subsoils of Haibei alpine grassland after incubation. Mean values are shown with standard error (n = 3). Bold dash and dotted lines represent mean values for all the top- and subsoils (n = 9), respectively. Different letters indicate significant difference between top- and subsoils (two-way ANOVA; $p < 0.05$).

induced vegetation changes on the Qinghai-Tibetan Plateau.

4.2. Limited microbial sequestration of freshly added carbon in the alpine grassland soils (especially the subsoil)

The long-term effect of substrate addition on soil carbon sequestration depends on the stability of retained new carbon versus that of primed SOC (Qiao et al., 2014). We therefore calculate CAE as amino sugars (representing microbial necromass) synthesized from freshly added carbon relative to metabolized fresh carbon to assess microbial efficiency of converting new carbon into stable SOC (necromass). CAE was lower in the subsoil compared to the topsoil of the Haibei alpine grassland ($p < 0.05$; Fig. 7), implying less efficient microbial sequestration of glucose derived carbon in the subsoil.

If we assume that amino sugars constitute a similar proportion of microbial biomass in soils from both depths, lower CAE in the subsoil hence suggests that microorganisms invest more carbon in catabolism than in anabolism (i.e., lower CUE) in the subsoil and/or that microbial residues are better preserved in the topsoil. The former mechanism is consistent with lower CUE with increasing OC degradation (Moorhead et al., 2013) and in nutrient-depleted subsoil (Manzoni et al., 2012). The latter mechanism, if present, is unlikely to be driven by mineral variables, given the higher clay content in the subsoil (Table 1) providing better physical protection for microbial necromass (Lehmann et al., 2007). Alternatively, higher CAE in the topsoil may be associated with a higher fungi-to-bacteria ratio (evidenced by the ratio of glucosamine:muramic acid; Fig. 6) compared with substrate-depleted subsoils (Fierer et al., 2003b; Struecker and Joergensen, 2015) because fungal residues (glucosamine) are considered to be relatively more stable than bacterial necromass (Indorf et al., 2015). Furthermore, the addition of large amounts of glucose to the subsoil may have exhausted the limited pool of bioavailable N in the Haibei alpine grassland (Xu et al., 2006; Zhou et al., 2014). As a result, muramic acid was most probably used as an N source to maintain microbial stoichiometry, resulting in its large decrease in the subsoil (Fig. 5c). Hence, N limitation induced by glucose amendment likely enhanced amino sugar decomposition in the subsoil relative to the topsoil (see Section 4.3), contributing to the lower CAE in the subsoil. Yet, this explanation should be taken with caution because SOC-normalized glucose amendment rate was much higher in the

Table 2
Isotopic composition, proportion of freshly added carbon (f_G) and glucose-derived amino sugars (AS_G) in soils (mean ± standard error). CK: control treatment, W1: whole-year warming, W2: enhanced winter warming.

	CK		W1		W2	
	Topsoil	Subsoil[a]	Topsoil	Subsoil	Topsoil	Subsoil
$\delta^{13}C$ (‰)						
Glucosamine	333 ± 5	1199 ± 206*	398 ± 72	1806 ± 254*	299 ± 30	1552 ± 155*
Galactosamine	276 ± 58	387 ± 32	344 ± 36	625 ± 216	187 ± 31	293 ± 6
f_G (%)[b]						
Glucosamine	0.004 ± 0.0001	0.014 ± 0.002*	0.005 ± 0.001	0.021 ± 0.003*	0.004 ± 0.0003	0.018 ± 0.002*
Galactosamine	0.003 ± 0.001	0.005 ± 0.0004	0.004 ± 0.0004	0.007 ± 0.002	0.002 ± 0.0003	0.004 ± 0.0001
AS_G (μg C g^{-1} soil)						
Glucosamine	0.047 ± 0.003	0.034 ± 0.008	0.050 ± 0.006	0.035 ± 0.001*	0.043 ± 0.002	0.030 ± 0.002*
Galactosamine	0.019 ± 0.005	0.006 ± 0.002*	0.022 ± 0.003	0.007 ± 0.002*	0.014 ± 0.001	0.003 ± 0.0004*
Total	0.066 ± 0.007	0.040 ± 0.006*	0.071 ± 0.009	0.042 ± 0.001*	0.057 ± 0.002	0.033 ± 0.002*

*indicates significant difference between top- and subsoils (*t*-test; $p < 0.05$).
[a] Two replicates for amino sugar analysis of this soil due to sample loss during transport. Three replicates for all other soils.
[b] The $\delta^{13}C$ values of glucosamine and galactosamine in non-amended soils are −30 and −31‰ in the topsoil and −27 and −26‰ in the subsoil, respectively.

subsoil than the topsoil, likely contributing to the intensified N limitation in subsoils. Lower CAE in the subsoil may hence partially result from our experiment design.

It must be noted that amino sugars are only part of the microbial necromass, although the exact proportion remains unknown. Hence the CAE we calculated may represent a lower limit of microbial carbon sequestration potential. In pure fungal and bacterial cultures, glucosamine and galactosamine are reported to have an average concentration of 47–49 and ~1.2 mg g^{-1}, respectively (reviewed by Appuhn and Joergensen, 2006; Engelking et al., 2007). Hence, glucosamine- and galactosamine-derived carbon makes up ~4% and 0.1% of fungal and bacterial biomass carbon, respectively (given a carbon content of 40% and 46% for amino sugars and microbial biomass, respectively). These ratios likely represent the minimum proportion of amino sugars in the total microbial carbon (biomass and necromass combined), as these compounds are considered to persist with microbial cell death and should make up a larger part of microbial necromass. Nonetheless, by the above ratios, the AS_G values of these two amino sugars (Table 2) correspond to a maximum total of 0.03 mg C g^{-1} soil of glucose-derived microbial carbon in all samples, if we assume that the newly synthesized glucosamine and galactosamine are exclusively derived from fungal and bacterial sources, respectively. Given the glucose amendment rate (1.4 mg C g^{-1} soil), only 2% of freshly added carbon was stabilized as microbial residues at the maximum, equivalent to an average of 2–6% of PE in this short-term experiment. Hence, accumulation of microbial necromass cannot counterbalance priming of native SOC in the Haibei alpine grassland. Long-term assessment of microbial necromass accumulation versus priming of stable SOC is needed to evaluate sequestration potentials of stable SOC pools following labile carbon addition to soils.

It should be pointed out that glucose is shown to be mainly used for catabolic processes rather than for microbial biomass build-up (Gunina et al., 2014), which may contribute to the low CAE in our experiment. Future investigations should employ more complexed substrates to examine the influence of substrate quality on microbial CAE. Also, results from our experiment should be considered to be a qualitative prediction of how SOC and fresh substrate decomposition would respond to increased plant inputs under warming at different soil depths. In the field where soil temperature, moisture and O_2 availability differ considerably from laboratory conditions, microbial processes may vary, leading to different SOC dynamics.

4.3. Enhanced decomposition of microbial necromass under N limitation

While glucosamine and galactosamine are considered to represent the relatively stable pool of microbial residues (Liang and Balser, 2012), bacteria-derived muramic acid is easy to degrade under substrate or N limitations (Engelking et al., 2008; Indorf et al., 2015). Soil microbes in the Haibei alpine grassland may be N-limited as microbial mineralization of N is constrained by low temperatures and there is a strong competition for available N with plants (Xu et al., 2006; Zhou et al., 2014). Moreover, glucose addition is reported to cause net immobilization of mineral N (Guenet et al., 2010; Paterson and Sim, 2013), further increasing N limitation during incubation. During our 86-day incubation, muramic acid decreased substantially in all glucose-amended soils while glucosamine and galactosamine remained constant (Fig. 5), reflecting the lability of muramic acid and probably N limitation caused by glucose-stimulated microbial growth and/or activity (Schneckenberger et al., 2008).

As > 90% of amino sugars are found in dead microbial cells in the soil (Amelung et al., 2001), the decrease of muramic acid was dominated by an enhanced cycling of labile components in microbial necromass in the original soil instead of faster turnover of microbial biomass, although the latter may have also occurred during priming (Blagodatskaya and Kuzyakov, 2008). Glucose addition likely enhanced the activity of extracellular enzymes including those associated with organic N degradation, as has been commonly reported in soils under increased labile carbon input (Weintraub et al., 2007; Dorodnikov et al., 2009), and thereby increased the turnover of labile microbial residues enriched in organic N in the original soil.

Compared with the non-amended incubation, glucose-amended subsoil witnessed a particularly large decrease of muramic acid (Fig. 5c), suggesting an increased N limitation in the deeper soils of Haibei alpine grassland. As mentioned previously, this may be related to a higher glucose amendment rate in the subsoil. Alternatively, a higher level of N limitation has also been reported at depth in arable (Struecker and Joergensen, 2015) and permafrost soils (Koven et al., 2015). It also implies that microbial necromass is more vulnerable to substrate-induced degradation in the subsoil. Our observation calls for a more accurate assessment of the stability of soil microbial residues with increased carbon inputs under warming or rising CO_2 levels (Kallenbach et al., 2015).

4.4. Reduced degradable SOC and increased N limitation in the subsoil under enhanced winter warming

In contrast to microbial residues, plant-derived DOC components are relatively labile and subject to rapid concentration changes due to temperature-induced variations in microbial activity and enzyme production processes (Creamer et al., 2015). In our experiment, dissolved lignin and R_S were lower in the subsoil of W2 relative to CK but not in the topsoil (Figs. 2 and 4b), indicating that enhanced winter warming reduced labile or degradable SOC in the subsoil without affecting that in the topsoil. This finding may be associated with the smaller size and/or a higher degradability of labile SOC pool in the subsoil, evidenced by a decreasing content of dissolved lignin in the CK and W1 subsoil after non-amended incubation in contrast with a constant level of dissolved lignin in the topsoil (Fig. 4). Dissolved lignin content was too low in the W2 subsoil (due to warming-induced microbial processing) to be further reduced during incubation, further confirming that enhanced seasonal warming diminished labile SOC.

Interestingly, glucose amendment increased dissolved lignin content (Fig. 4b) in parallel with a significant decline of amino sugars (microbial necromass) in the W2 subsoil (Fig. 5d) while such changes were not observed in the CK or W1 subsoil. As discussed previously, the decrease of amino sugars is an indicator of elevated N limitation in the W2 subsoil. In the W2 subsoil that experienced a higher degree of seasonal warming and nutrient limitation, glucose addition likely enhanced microbial enzyme production or activity to alleviate microbial nutrient stress (Kuzyakov et al., 2000; Craine et al., 2007; Kuzyakov, 2010). Consequently, dissolved lignin production increased due to enzymatic oxidation of the macromolecular (undissolved) lignin and positive priming was enhanced in the soil. In other words, an increasing concentration of dissolved lignin may be accompanied by elevated decomposition of the lignin macromolecule. This explanation is supported by the stimulated activity of phenol oxidase (a key enzyme involved in lignin degradation) in nutrient-limited soils during priming with sucrose but not in combination with nutrients or in nutrient-rich soils (Nottingham et al., 2012). It is also consistent with findings by Zhang et al. (2015), where glucose addition to a deep soil primed the decomposition of aromatic C-O structures observed by nuclear magnetic resonance spectroscopy.

In summary, the Haibei alpine grassland subsoil was depleted of

labile SOC (assessed by R_S and dissolved lignin content) under elevated seasonal warming and exhibited intensified N limitation and higher relative PE under labile carbon additions. Compared with the topsoil, a higher fraction of freshly added carbon is used for microbial catabolism while a smaller proportion is stabilized as microbial residues in the subsoil, leading to a lower CAE at depth. These results seem to indicate a higher vulnerability of subsoil SOC to warming-induced substrate changes in the Haibei alpine grasslands. Alternatively, it has previously been demonstrated that higher PE can be induced by higher labile carbon additions per SOC (Paterson and Sim, 2013; Rousk et al., 2015). As such, it is also possible that the higher glucose addition rate (relative to SOC) contributed to the higher relative PE and lower CAE in the subsoils. Mechanisms controlling the distinct response of subsoil SOC to substrate increases warrant further investigation to better predict climate warming effect on the soil carbon pool on the Qinghai-Tibetan Plateau.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.10.018.

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