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PRIMARY RESEARCH ARTICLE

Inactive and inefficient: Warming and drought effect on microbial carbon processing in alpine grassland at depth

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Abstract

Subsoils contain >50% of soil organic carbon (SOC) globally yet remain underinvestigated in terms of their response to climate changes. Recent evidence suggests that warmer, drier conditions in alpine grasslands induce divergent responses in SOC decomposition and carbon accrual in top- versus subsoils. However, longer term effects on microbial activity (i.e., catabolic respiration vs. anabolic growth) and belowground carbon cycling are not well understood. Here we utilized a field manipulation experiment on the Qinghai-Tibetan Plateau and conducted a 110-day soil incubation with and without ^{13}C-labeled grass litter to assess microbes' role as both SOC "decomposers" and "contributors" in the top- (0–10 cm) versus subsoils (30–40 cm) after 5 years of warming and drought treatments. Microbial mineralization of both SOC and added litter was examined in tandem with potential extracellular enzyme activities, while microbial biomass synthesis and necromass accumulation were analyzed using phospholipid fatty acids and amino sugars coupled with ^{13}C analysis, respectively. We found that warming and, to a lesser extent, drought decreased the ratio of inorganic nitrogen (N) to water-extractable organic carbon in the subsoil, intensifying N limitation at depth. Both SOC and litter mineralization were reduced in the subsoil, which may also be related to N limitation, as evidenced by lower hydrolase activity (especially leucine aminopeptidase) and reduced microbial efficiency (lower biomass synthesis and necromass accumulation relative to respiration). However, none of these effects were observed in the topsoil, suggesting that soil microbes became inactive and inefficient in subsoil but not topsoil environments. Given increasing belowground productivity in this alpine grassland under warming, both elevated root deposits and diminished microbial activity may contribute to new carbon accrual in the subsoil. However, the sustainability of plant growth and persistence of subsoil SOC pools deserve further investigation in the long term, given the aggravated N limitation at depth.

KEYWORDS
carbon utilization efficiency, climate change, deep soil, microbial necromass, mineralization potential, nitrogen limitation, soil organic carbon

Erxiong Zhu and Zhenjiao Cao contributed equally to this work.

1 | INTRODUCTION

The response of soil organic carbon (SOC) to global warming and warming-induced soil drying (Dai, 2013) has a critical impact on atmospheric carbon dioxide levels and the trajectory of future climate change (Melillo et al., 2017). While most previous studies have focused on topsoil carbon dynamics (Crowther et al., 2016; Koven et al., 2017), subsoils, which are >20 cm belowground and contain >50% of global SOC stocks (Rumpel et al., 2012), are currently drawing much attention (Hicks Pries et al., 2017; Jia et al., 2019). Several emergent studies suggest that subsoil SOC may show distinct responses to warming and drought compared with topsoil (Fierer et al., 2003; Jia et al., 2019), depending on vegetation, microbial and soil nutrient shifts (Liu et al., 2018; Salomé et al., 2010). Hence, it is essential to elucidate mechanisms underlying the top-subsoil contrasts in order to better understand and predict SOC variations under climate change.

The world's highest and largest plateau, Qinghai-Tibetan Plateau (QTP), harbors a vast area of alpine grasslands that experience a warming trend of 0.2°C per decade (Chen et al., 2013), accompanied by decreasing rainfall in most regions in recent years (Xu et al., 2008). Warming and/or drought is reported to decrease soil moisture, increasing the relative abundance of deep-rooted grasses in the plant community and increasing belowground net primary productivity (BNPP) in the QTP alpine grassland studied here (Liu et al., 2018, 2020). Recently, we have also observed warming-induced changes in carbon allocation in the subsoil (but not topsoil) of the QTP alpine grassland, including enhanced accumulation of newly synthesized carbon in the fine-sized subsoil fraction (Jia et al., 2019). While this observation is considered to be closely related to deeper root distribution under warming and drying, how microbial processes respond and contribute to the altered subsoil carbon dynamics remains to be explored.

Soil microbes may regulate SOC dynamics via at least three pathways. First, microbes decompose SOC via secreting carbon-degrading extracellular enzymes and mineralizing organic substrates into greenhouse gases (Bond-Lamberty et al., 2018; Chen et al., 2020). Second, microbes mediate soil nutrient cycling by secreting nitrogen (N)- and phosphorus (P)-acquiring enzymes, which in turn affects plant growth (SOC inputs) and microbial activity (Burke et al., 2011; Cheeke et al., 2017; Sinsabaugh et al., 2009). Third, microbes contribute to SOC formation by converting degradable organic matter into microbial necromass and by-products via iterative turnover of the living community, which tend to accrue in the relatively slow-cycling, fine-sized soil fraction and contribute to soil carbon sink potentials in the long term (Cotrufo et al., 2013; Kallenbach et al., 2016; Liang et al., 2017, 2019; Sokol & Bradford, 2018).

In the QTP alpine grassland, deeper root distribution under warming and drought is considered to enhance carbon inputs into the subsoil as root deposits (Liu et al., 2018, 2020), potentially fueling the activity of carbon-deprived subsoil microbes (Fontaine et al., 2007) and thus enhancing the role of microbes as both SOC "decomposers" and "contributors." Alternatively, alpine soils are often N-limited due to temperature-constrained mineralization of organic N (Xu et al., 2006). Increased root mass may strengthen nutrient competition between plants and microbes and intensify N limitation at depth (Hill & Jones, 2019; Jia et al., 2017; Liu et al., 2018). While mild N limitation may stimulate microbial secretion of N-acquiring enzymes (Cui et al., 2020), strong N (as well as moisture) limitation can inhibit microbial activity and enzyme synthesis (Allison & Vitousek, 2005; Schimel & Weintraub, 2003), thereby constraining microbes' role as "decomposer" and "contributor" of SOC (Anthony et al., 2020; Bicharanloo et al., 2020; Cui et al., 2020). Moreover, under nutrient deprivation, microbes tend to invest more in catabolism (respiration) rather than in anabolism (biomass synthesis), thus decreasing microbial carbon use efficiency (CUE; Spohn et al., 2016) and microbial carbon accumulation efficiency (CAE; Jia et al., 2017) and undermining the overall efficiency of soil to accumulate microbial-derived, slow-cycling carbon in the long term. These two scenarios have distinct consequences for soil carbon cycling and hence deserve investigation.

To test which of the above scenarios follow warming and drought in the QTP alpine grassland, we employ a soil incubation experiment with and without the amendment of ^{13}C-labeled grass litter (leaf or root) to the topsoil (0–10 cm) and subsoil (30–40 cm) collected from the same QTP field manipulation experiment after 5 years of warming and drought treatments (Jia et al., 2019). Microbial capacity of carbon mineralization is examined using the mineralization potential of both SOC (R_{SOC}) and added litter (R_{litter}) under optimal temperature and moisture conditions (Shaver et al., 2006), in combination with extracellular enzyme activity assays. Microbial synthesis of biomass and necromass is analyzed in tandem using phospholipid fatty acids (PLFAs; Frostegård & Bååth, 1996) and amino sugars (Amelung, 2001) coupled with ^{13}C analysis, respectively, facilitating the assessment of CUE and CAE in the soil. Coupled with soil bulk chemistry analysis (including carbon and N availability), we aim to elucidate and compare the mechanisms driving changes in microbial carbon processing in the top- versus subsoils of the QTP alpine grassland.

2 | MATERIALS AND METHODS

2.1 | Field manipulation experiment and soil sampling

The field manipulation experiment is located at the Haibei Alpine Grassland Ecosystem Research Station on the northeastern edge of QTP (101°19′E, 37°36′N, 3215 m a.s.l.; Figure S1a). The region has a continental monsoon climate with a mean annual temperature of −1.2°C and a mean annual precipitation of 489 mm. Soils in this area are Mat-Gryic Cambisol according to the IUSS Working Group World Reference Base for Soil Resources (WRB, 2015) with a clay loam texture (Jia et al., 2017). The native plant community is dominated by *Kobresia humilis*, *Carex przewalskii*, *Stipa aliena*, *Saussurea pulchra*, and *Elymus nutans* (Liu et al., 2018).

The experiment had a full factorial design with six replicated plots set up in July 2011, including whole-year warming via infrared heating devices (installed 1.6 m above the soil surface), precipitation reduction by 50% using polyvinyl chloride baffles (drought) and ambient treatments (in a natural state; Figure S1b). On an annual average basis, experimental warming increased surface soil temperatures by 1.5–1.8°C and reduced soil moisture by 12% relative to the ambient treatment. The experimental drought treatment reduced soil moisture at 20 cm depth by 20% relative to the ambient treatment (Liu et al., 2018). After both warming and drought treatments, the relative abundance of deep-rooted grasses increased while sedges and forbs with relatively shallower roots decreased, resulting in increased BNPP relative to the ambient soils at depths (Liu et al., 2018, 2020).

After 5 years of experiment, three replicate plots were sampled within each field treatment at the end of the growing season (August 2015). Soils were randomly collected using a corer (diameter of 3 cm) from 0–10 cm (i.e., topsoil) and 30–40 cm (i.e., subsoil) in each plot, immediately shipped back to the laboratory, and passed through a 2-mm sieve with visible roots manually removed. Subsequently, the soils were stored in two parts: one part was air-dried to determine soil physicochemical properties, and the other part was stored at 4°C before the incubation experiment (for <30 days).

2.2 | Soil physicochemical analyses

Basic soil physicochemical properties were determined for the air-dried original soil. SOC and soil nitrogen (SN) contents were determined by an elemental analyzer (Vario EL III; Elementar), with inorganic carbon removed by fumigation with concentrated hydrochloric acid (HCl) before SOC measurement (Harris et al., 2001). Water-extractable organic carbon (WEOC) was extracted by vortexing 1.5–2.0 g ground soil (ball milled to ~2500 mesh) in 12 ml Milli-Q water for 1 min (Zhu et al., 2020). The supernatant was filtered by pre-rinsed 0.45-μm poly(tetrafluoroethylene) syringe filters and acidified (pH <2) before measurement on a multi N/C 3100 total organic carbon analyzer (Analytik Jena). Soil inorganic N (IN) including nitrate (NO_3^-) and ammonium (NH_4^+) was extracted by 0.01 M calcium chloride in a soil-to-solution ratio of 1:10 (w:v) and determined by a flow analyzer (AA3, SEAL). The ratio of IN to WEOC was used to assess the availability of IN relative to available carbon in the soil (Taylor & Townsend, 2010; Wickland et al., 2012).

Given that oxygen diffusion may be limited in the subsoil (Xiang et al., 2008), thereby constraining microbial activity (Hicks Pries et al., 2016), we also measured concentrations of soil ferrous iron [Fe(II)] and ferric Fe [Fe(III)], and their ratio [Fe(II)/Fe(III)], to assess soil redox conditions in the field (Hall & Silver, 2015). Fresh soils were taken from the same depth outside the treatment plots (n = 4) and immediately immersed in 0.5 M HCl for 24 h in August 2015. After centrifugation, Fe(II) in the supernatant was measured by absorbance at 562 nm on an ultraviolet and visible spectrometer (Shimadzu UV-2550) after mixing with 5 mM ferrozine solution (Stookey, 1970). Total Fe was first reduced using hydroxylamine hydrochloride (2%) and then analyzed as Fe(II) as above. Fe(III) was calculated by subtracting Fe(II) from total Fe.

2.3 | Soil incubation experiment

To examine microbial capacity for carbon mineralization (mineralization potentials) and necromass accumulation, soils were incubated with and without the addition of ^{13}C-labeled grass leaf or fine root (<2 mm) litter of *Oplismenus undulatifolius* and *Miscanthus sinensis* mixture in fine powders (ball milled to ~2500 mesh). These grass litters were used to represent the vast majority of litter inputs (including fine roots) that turn over relatively fast in grasslands (Solly et al., 2014) while ^{13}C-labeled local vegetation was not commercially available. Specifically, ~15 g (dry weight) fresh top- and subsoils (stored at 4°C) from the ambient, drought and warming treatments were placed separately in 165-ml brown culture flasks and adjusted to 60% of the soil's maximum water holding capacity. All samples were pre-incubated at 25°C for 1 week in the dark to activate soil microbes. Subsequently, soils were divided into three amendment groups: the first group received no exogenous carbon (control); the second group received ^{13}C-labeled leaves (δ^{13}C: 2067.8‰, carbon content: 41.6%, C/N ratio: 18.6; Table S1); the third group received ^{13}C-labeled roots (δ^{13}C: 2519.1%, carbon content: 41.4%, C/N ratio: 34.0; Table S1). The amount of added carbon was ~0.7% of SOC for the top- and subsoils and no priming effect was induced (Section 3.2). In total, 54 flasks were prepared, including three replicated plots from three field treatments, three amendments, and two soil depths.

The incubation started immediately after exogenous carbon addition. All flasks were kept in the dark at 25°C for 110 days. The incubation was used to measure "specific potential heterotrophic respiration," which is calculated as the potential maximum of heterotrophic respiration measured at 25°C and at 60% water holding capacity, normalized to the OC content of bulk soil and litter, respectively (Doetterl et al., 2018). Deionized water was regularly sprayed to maintain a constant soil moisture content. Respiration was measured on Days 1, 3, 6, 10, 15, 23, 33, 46, 52, 65, 83, 91, and 110 by quantifying CO_2 on a gas chromatograph (GC; Agilent 7890A) coupled with a flame ionization detector. The δ^{13}C of respired CO_2 was measured periodically (five times in total) on an isotope ratio mass spectrometer (IRMS; GasBench II, Delta PLUS Advantage, Thermo Finnigan), and SOC- versus litter-derived CO_2 was estimated using the following mass balance equations:

$$r_T = r_{SOC} + r_{litter}, \quad (1)$$

$$r_T \times \delta^{13}C_T = r_{SOC} \times \delta^{13}C_{SOC} + r_{litter} \times \delta^{13}C_{litter}, \quad (2)$$

where r_T, r_{SOC} and r_{litter} are the cumulative CO_2 (mg C g^{-1} soil) respired from the bulk sample, derived from SOC and from litter (leaf or root), respectively; $\delta^{13}C_T$, $\delta^{13}C_{SOC}$, $\delta^{13}C_{litter}$ are the δ^{13}C values of respired CO_2, SOC, and added litter, respectively. The mineralization potentials

(expressed in %) of SOC (R_{SOC}) and litter (R_{litter}) were calculated by normalizing r_{SOC} and r_{litter} to the corresponding organic carbon content of soil and litter, respectively.

2.4 | Assay of extracellular enzyme activity

The activity of one major oxidase (phenol oxidase) and four major hydrolases (α-glucosidase, β-glucosidase, alkaline phosphatase, and leucine aminopeptidase) related to carbon, N, and P hydrolysis were measured at the end of the incubation (Saiya-Cork et al., 2002). Briefly, 1 g of fresh soil was added to 91 ml Milli-Q water and homogenized with a magnetic stirrer for 3 min. For the hydrolases, the resulting suspension (200 μl) was dispensed into 96-well microplates with 50 μl of 4-methylumbelliferone for α-glucosidase, β-glucosidase, and alkaline phosphatase, or 7-amino-4-methylcoumarin for leucine aminopeptidase in pH buffers. The microplates were incubated in the dark at 25°C for 4 h. For phenol oxidase, 50 μl of L-3,4-dihydroxyphenylalanine (5 mM) in Tris buffer solutions (pH of 7.8) was added to each sample well and incubated in the dark at 25°C for 3 h. Eight replicate wells were used per sample per assay. Potential enzyme activity was quantified using Multi-Mode Microplate Reader (synergy Mx; BioTek Instruments Inc.). Fluorescence (for hydrolases) was measured with excitation at 365 nm and emission at 450 nm, while absorbance (for phenol oxidase) was measured at 450 nm. The SOC-normalized specific activity of enzymes was expressed in units of μmol g^{-1} SOC h^{-1} or mmol g^{-1} SOC h^{-1}.

2.5 | Phospholipid fatty acid analysis

Microbial community structure and biomass were analyzed by PLFAs using a modified Bligh–Dyer method at the end of the incubation and transformed into fatty acid methyl esters (FAMEs; details in Supplementary Methods; Bligh & Dyer, 1959; Cao et al., 2019). FAMEs were identified and quantified against internal standards on a Trace 1310 gas chromatograph coupled with an ISQ mass spectrometer (GC/MS; Thermo Fisher Scientific) using a DB-5MS capillary column (30 m × 0.25 mm ×0.25 mm) for separation (details in Cao et al., 2019). Fatty acids are designated according to the standard PLFA nomenclature (Guckert et al., 1985). Gram-positive (G+) bacteria are represented by PLFAs *i*15:0, *a*15:0, *i*16:0, *i*17:0, and *a*17:0, while Gram-negative (G−) bacteria are represented by PLFAs *cy*17:0 and 19:1ω8*c*. Fungi-specific PLFA (18:1ω9*c*) is also summarized (Harwood & Russell, 1984). Microbial community composition was assessed by ratios of G+ to G− bacteria (G+/G−) and fungi to bacteria (F/B). Microbial biomass is represented by total PLFAs (Cao et al., 2019), including all identified PLFAs (C_{14}–C_{19}, 20:0), and normalized to the SOC content.

2.6 | Amino sugar analysis

Microbial necromass was assessed by amino sugars at the end of the incubation using HCl hydrolysis (details in Supplementary Methods Glaser et al., 2004; Zhang & Amelung, 1996). Amino sugar derivatives were identified on GC/MS similar to PLFAs with a different oven temperature program (Ma et al., 2018). Quantification was achieved by comparing with internal standards to account for compound loss during extraction procedures. External quantification standards were used to normalize the response factor for different amino sugars. Amino sugars were summarized as glucosamine, galactosamine, mannosamine, and muramic acid. Among them, glucosamine is more abundant in fungal than bacterial cell walls while muramic acid is considered to trace bacteria-derived carbon (Guggenberger et al., 1999; Liang et al., 2008). The other two amino sugars are ubiquitous.

2.7 | Compound-specific ^{13}C isotopic analysis and calculations

To differentiate microbial biomass and necromass synthesized from SOC versus litter carbon, the ^{13}C isotopic composition of individual PLFAs (i.e., FAMEs) and two major amino sugar derivatives with sufficient abundances (glucosamine and galactosamine) was analyzed on a Thermo Trace GC Ultra coupled to a stable isotope ratio mass spectrometry (Thermo MAT 253) via a combustion interface (GC-C-IRMS) using similar conditions as the GC/MS analysis. The $\delta^{13}C$ values of PLFAs and amino sugars were corrected for the derivative carbon added to each molecule using a mass balance approach (Cao et al., 2019; Denef et al., 2009; Jia et al., 2017). For each soil sample, the $\delta^{13}C$ of total PLFAs was estimated by the abundance-weighted average of the 10 most abundant PLFAs. The selected PLFAs represented ~42% of total PLFAs in all samples and included G+ bacterial (*i*15:0, *a*15:0, *i*16:0, *i*17:0, and *a*17:0), G− bacterial (19:1ω8*c* and *cy*17:0), and fungal PLFAs (18:1ω9*c*). The abundance-weighted average $\delta^{13}C$ of amino sugars was also calculated based on glucosamine and galactosamine.

The contribution of litter-derived carbon to total PLFAs or amino sugars was calculated as follows:

$$f_{litter} = \frac{\delta^{13}C_C - \delta^{13}C_{SOC}}{\delta^{13}C_{litter} - \delta^{13}C_{SOC}} \times 100\%, \quad (3)$$

where f_{litter} is the percentage of compounds derived from ^{13}C-labeled litter and $\delta^{13}C_C$ represents the abundance-weighted average $\delta^{13}C$ for the target compounds.

Microbial CUE was then calculated based on PLFAs (referred to as CUE′ to differentiate from microbial biomass carbon-based CUE; Cao et al., 2019; Kallenbach et al., 2016):

$$CUE' = \frac{PLFA - C_{litter}}{PLFA - C_{litter} + CO_2 - C_{litter}} \times 100\%, \quad (4)$$

where PLFA-C_{litter} and CO_2-C_{litter} are the amount of carbon in litter-derived PLFAs (multiplying f_{litter} with PLFA concentrations and the carbon content of PLFAs) and CO_2, respectively (Bradford et al., 2013).

Microbial necromass accumulation efficiency (CAE) was similarly calculated, reflecting how efficiently microbes convert degradable carbon into necromass (Jia et al., 2017):

$$CAE = \frac{\text{Amino sugar} - C_{litter}}{\text{Amino sugar} - C_{litter} + CO_2 - C_{litter}} \times 100\%, \quad (5)$$

where amino sugar-C_{litter} is the amount of carbon in litter-derived amino sugars, calculated by multiplying f_{litter} with amino sugar concentrations and the carbon content of amino sugars (40%; Jia et al., 2017).

2.8 | Statistical analysis

All statistical analyses were conducted using SPSS 20 (SPSS) or R version 3.6.1 (R Development Core Team, 2017). Normal distribution of data and homogeneity of variance were checked using Shapiro–Wilk and Levene's tests, respectively. To compare the effect of soil depth and field treatments (ambient, drought, and warming) on soil properties before the incubation experiment, two-way ANOVA was used. Three-way ANOVA was used to analyze the effect of soil depth, field treatments, and litter amendments (control, leaf, and root additions) on parameters in the incubation experiment, where three-way interactions were not observed except for phenol oxidase activity and CUE′. In the presence of interactive effects between two factors, two-way ANOVA was further used to distinguish the influence of different factors. To ascertain the influence of a single factor, one-way ANOVA and Kruskal–Wallis tests were used for the normally and non-normally distributed data, respectively. Repeated-measures ANOVA was also used to confirm field treatment effects on soil respiration at different sampling times. To probe environmental influences on microbial-related parameters, Pearson and Spearman correlations were used for the normally and non-normally distributed data, respectively. To verify the key environmental influence(s) on microbial parameters, multiple linear regression models were performed using the "lm" function in R, incorporating main variables showing significant correlations with the microbial parameters. Differences and correlations were considered to be significant at a level of $p < 0.05$.

3 | RESULTS

3.1 | Field soil properties

The Fe(II)/Fe(III) ratio was significantly higher in the top- (0.35 ± 0.08) than subsoil at the Haibei Station (0.18 ± 0.03; $n = 4$; $p < 0.05$), suggesting that subsoil microbes were not significantly constrained by anaerobiosis at the investigated depth. Given the improved aeration with decreased soil moisture under warming and drought, anaerobiosis was even less likely in the subsoils under the field treatments. The topsoil had higher SOC, SN, and WEOC than the subsoil ($p < 0.05$; Figure 1a–e; Table S1). Over 5 years of field treatments had no significant effect on the SOC, SN, or WEOC in the topsoil ($p > 0.05$), but increased WEOC (under warming) and SN contents (under both warming and drought) in the subsoil ($p < 0.05$). Both warming and drought decreased soil IN (inorganic nitrogen) in the topsoil ($p < 0.05$), but did not affect the ratio of IN/WEOC ($p > 0.05$; Figure 1d–f; Table S1). In contrast, both NO_3^- (the dominant form of IN) and the IN/WEOC ratio

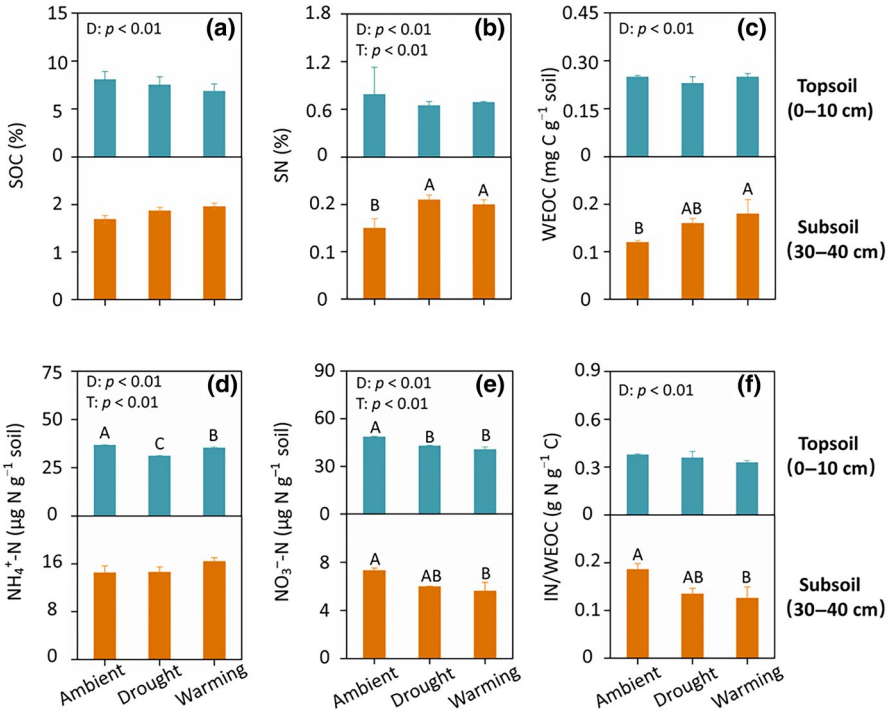

FIGURE 1 Field soil properties at the Haibei Station before the incubation experiment: (a) soil organic carbon (SOC); (b) soil nitrogen (SN); (c) water-extractable organic carbon (WEOC); (d) ammonium (NH_4^+) nitrogen; (e) nitrate (NO_3^-) nitrogen; (f) inorganic nitrogen (IN; including NH_4^+ and NO_3^-) to WEOC ratio. Mean values are shown with standard error ($n = 3$). Different uppercase letters denote different levels among field treatments ($p < 0.05$). D and T denote the effects of soil depth and field treatments, respectively (only significant effects based on two-way ANOVA are shown)

significantly decreased in the subsoil under warming relative to the ambient ($p < 0.05$).

3.2 | Carbon mineralization

In the 110-day incubation, both R_{SOC} and R_{litter} were significantly lower in the top- (1.9%–3.5% and 18.4%–29.8%, respectively) than subsoils (4.3%–7.2% and 30.3%–62.3%, respectively; $p < 0.05$; Figure 2). There was no significant difference in R_{SOC} among different amendments at either depth ($p > 0.05$), implying no priming effect. R_{litter} was higher in the leaf than root amendments in the subsoil ($p < 0.05$) but was similar for both amendments in the topsoil ($p > 0.05$). Compared to the ambient, both drought and warming decreased R_{SOC} and R_{litter} in the subsoil ($p < 0.05$) but not in the topsoil ($p > 0.05$). These results were consistent across time, as supported by the repeated-measures ANOVA of respiration rates calculated for different time points during the incubation (Figure S2).

3.3 | Extracellular enzyme activity

The SOC-normalized specific activity of all tested enzymes was significantly higher in the subsoil than topsoil at the end of the incubation ($p < 0.05$; Figure 3). Enzyme activity did not differ among different amendments ($p > 0.05$) except for a lower phenol oxidase activity in the subsoil with both leaf and root amendments than the control ($p < 0.05$; Figure 3e). Compared to the ambient treatments,

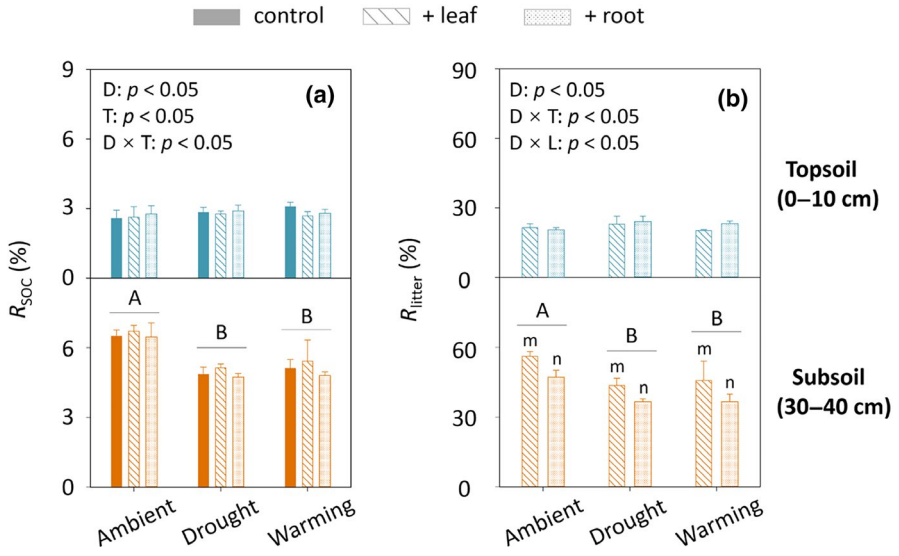

FIGURE 2 The mineralization potential of soil organic carbon (R_{SOC}; a) and litter carbon (R_{litter}; b) in the 110-day incubation experiment. Mean values are shown with standard error ($n = 3$). Different upper- and lowercase letters indicate different levels among field treatments and litter amendments, respectively ($p < 0.05$). D, T, and L denote the effects of soil depth, field treatments, and litter amendments, respectively (only significant effects based on three-way ANOVA are shown)

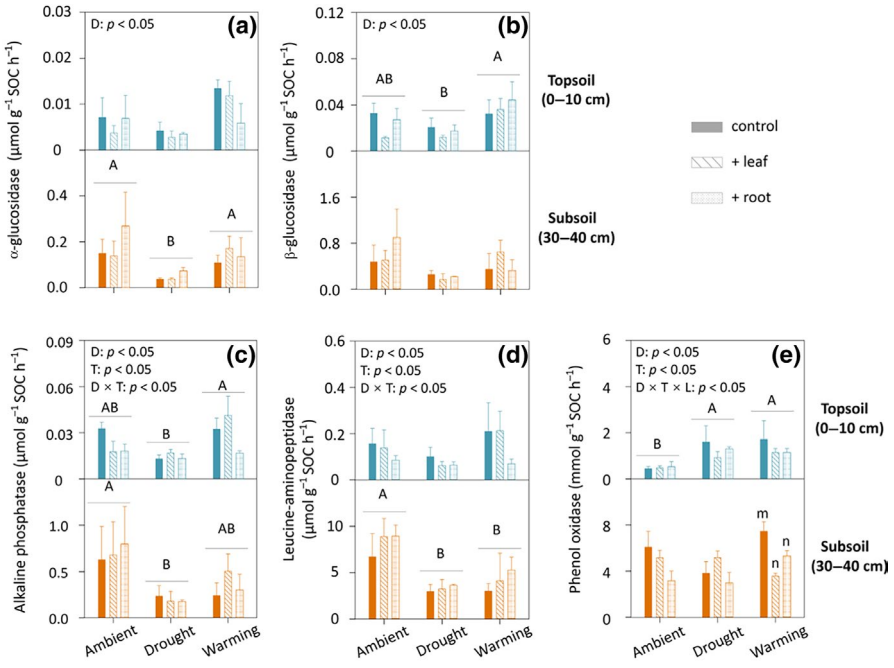

FIGURE 3 Soil organic carbon (SOC)-normalized specific activity of extracellular enzymes at the end of the incubation experiment: (a) α-glucosidase; (b) β-glucosidase; (c) alkaline phosphatase; (d) leucine aminopeptidase; (e) phenol oxidase. Mean values are shown with standard error ($n = 3$). Different upper- and lowercase letters indicate different levels among different field treatments and litter amendments, respectively ($p < 0.05$). D, T, and L denote the effects of soil depth, field treatments, and litter amendments, respectively (only significant effects based on three-way ANOVA are shown)

both drought and warming increased phenol oxidase activity in the topsoil and decreased leucine aminopeptidase activity in the subsoil ($p < 0.05$). Drought also decreased α-glucosidase and alkaline phosphatase activity in the subsoil relative to the ambient ($p < 0.05$).

3.4 | Microbial PLFAs and CUE′

At the end of the incubation, subsoils had higher SOC-normalized concentrations of total PLFAs and G+/G− ratios but lower F/B ratios relative to the topsoil ($p < 0.05$; Figure 4a–c). Leaf amendment increased the G+/G− ratio in the ambient topsoil relative to control and root amendment ($p < 0.05$). There was no significant difference in total PLFAs (0.002–0.3 mg g^{-1} SOC), F/B, or G+/G− ratios ($p > 0.05$) in soils under different field treatments. On average, 2.31%–3.38% and 0.85%–1.64% of PLFAs were derived from ^{13}C-labeled litter (both leaf and root) in the top- and subsoils, respectively (Table S2), resulting in lower CUE′ in the subsoil (0.02%–0.20%) than topsoil (0.09%–0.32%; $p < 0.05$; Figure 4d). CUE′ was similar in root and leaf amendments in the topsoil ($p > 0.05$) but higher in the leaf than root amendment in the subsoil ($p < 0.05$). Both warming and drought decreased CUE′ relative to the ambient in the subsoil ($p < 0.05$) but not topsoil.

3.5 | Amino sugars and CAE

Total amino sugars were 0.51–12.39 mg g^{-1} SOC at the end of the incubation, and glucosamine accounted for ~75 ± 2% of all amino sugars (Figure 4e; Figure S3). Litter amendments did not significantly influence the abundance or composition of amino sugars. Warming significantly reduced amino sugars in the topsoil relative to the ambient and drought, while both drought and warming decreased amino sugars in the subsoil relative to the ambient ($p < 0.05$; Figure 4e). Less than 1% of amino sugars were derived from litter after the incubation (Table S3). Microbial CAE was on average ~3 times higher in the topsoil (0.79 ± 0.12%) than in the subsoil (0.24 ± 0.08%; $p < 0.05$; Figure 4f). Microbial CAE decreased in the topsoil by 4–5 times under warming relative to the ambient and decreased by ~7 and 25 times in the subsoil under drought and warming, respectively ($p < 0.05$).

3.6 | Influencing factors on microbial parameters

To reveal drivers of changing microbial processes, we examined correlations between soil variables and the microbial parameters (including R_{SOC}, R_{litter}, CUE′, and CAE). Due to collinearity among some pairs of variables (such as the specific activity of hydrolases at both depths; $p < 0.05$; Figure S4), variables showing the strongest

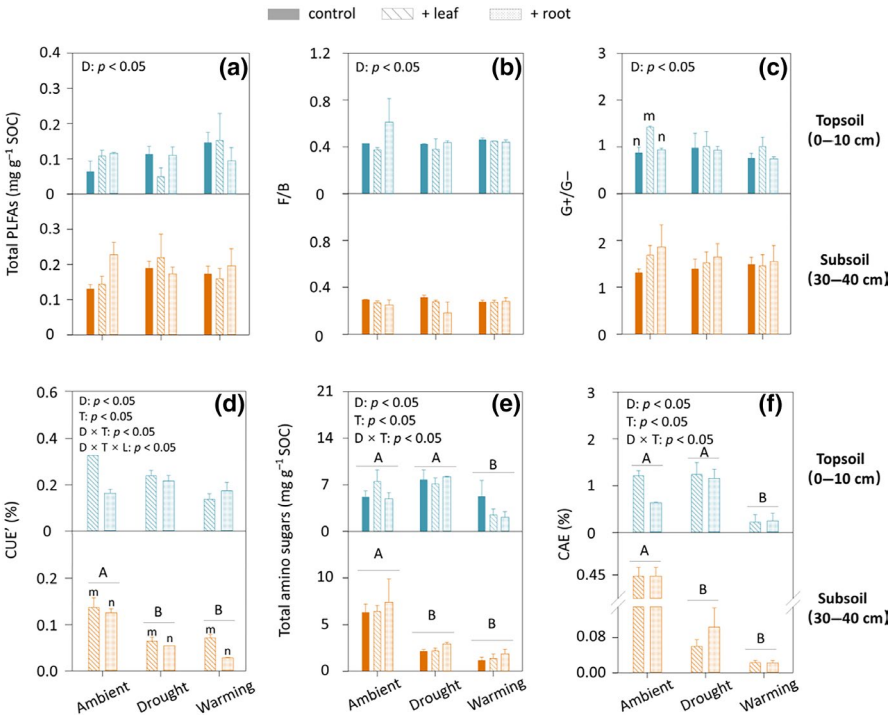

FIGURE 4 Parameters related to microbial phospholipid fatty acids (PLFAs) and amino sugars at the end of the incubation experiment: (a) total PLFAs in soils; (b) the ratio of fungal to bacterial PLFAs (F/B); (c) the ratio of gram-positive (G+) to gram-negative (G−) bacterial PLFAs (G+/G−); (d) PLFA-based microbial carbon use efficiency (CUE′); (e) total amino sugars; (f) microbial carbon accumulation efficiency (CAE). Mean values are shown with standard error ($n = 3$ except in (d) where two replicates for δ^{13}C-PLFA analysis were lost for the ambient topsoil with leaf amendment and drought subsoil with root amendment). Different upper- and lowercase letters indicate different levels among different field treatments and litter amendments, respectively ($p < 0.05$). D, T, and L denote the effects of soil depth, field treatments, and litter amendments, respectively (only significant effects based on three-way ANOVA are shown)

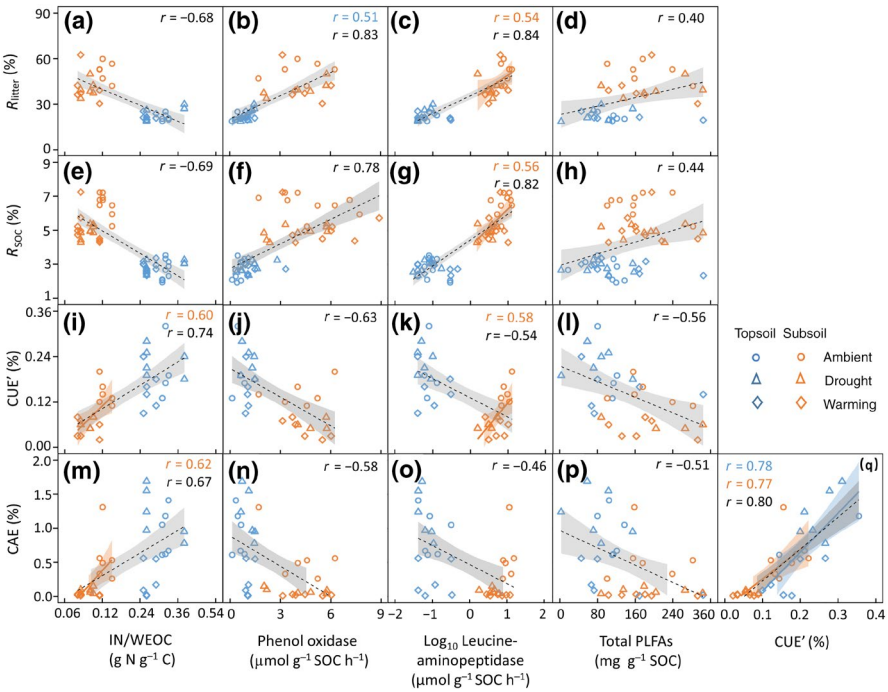

FIGURE 5 Relationships for the mineralization potential of soil organic carbon (R_{SOC}; a–d) and litter (R_{litter}; e–h), microbial carbon utilization efficiency (CUE′; i–l) and microbial carbon accumulation efficiency (CAE; m–q) with the main influencing variables. Blue, orange, and black lines represent correlations in the topsoil, subsoil, and across depths, respectively ($p < 0.05$). Shaded areas represent the 95% confidence intervals for the regression lines. IN, inorganic nitrogen; PLFAs, phospholipid fatty acids; WEOC, water-extractable organic carbon

correlations with the investigated parameters (i.e., leucine aminopeptidase) are shown in Figure 5.

R_{SOC} and R_{litter} were highly correlated in all field treatments and across depths ($r > 0.80$; $p < 0.05$), suggesting similar controls on SOC and litter mineralization. Both R_{SOC} and R_{litter} decreased with increasing IN/WEOC ratios across depths ($r = -0.68$ to -0.69; $p < 0.05$), but the relationship seemed to result from contrasting values of variables between depths and was not present at either depth separately. R_{SOC} and R_{litter} increased with increasing phenol oxidase activity across depths ($r = 0.78$–0.83; $p < 0.05$), leucine aminopeptidase activity in the subsoil ($r = 0.54$–0.56), and across depths ($r = 0.82$–0.84; $p < 0.05$), and to a lesser extent, concentrations of total PLFAs across depths ($r = 0.40$–0.44; $p < 0.05$; Figure 5a–h). Multiple linear regression models confirmed that only leucine aminopeptidase activity had a significant effect on R_{SOC} and R_{litter} when all of the above variables were incorporated into the model ($p < 0.001$; Table S4), suggesting its dominant influence on the mineralization potentials.

Microbial CUE′ and CAE were strongly correlated in all soils ($r = 0.80$; $p < 0.05$; Figure 5q). Both were strongly correlated with the IN/WEOC ratio across depths ($r = 0.67$–0.74; $p < 0.05$) and in the subsoil ($r = 0.60$–0.62; $p < 0.05$). CUE′ and CAE decreased with increasing phenol oxidase and leucine aminopeptidase activity across depths ($r = -0.46$ to -0.63; $p < 0.05$), but the relationships also seemed to result from contrasts between depths and were absent at either depth separately. On the contrary, CUE′ increased with increasing leucine aminopeptidase activity in the subsoil ($r = 0.58$; $p < 0.05$) while both CUE′ and CAE decreased with total PLFAs across depths ($r = -0.51$ to -0.56; $p < 0.05$; Figure 5i–p). Multiple linear regression models confirmed that only the IN/WEOC ratio had a significant effect on both CUE′ and CAE ($p < 0.01$; Table S4), suggesting the dominant influence of N availability on microbial carbon processing efficiencies.

4 | DISCUSSION

In the investigated alpine grassland, we find that 5 years of field warming and drought treatments have led to striking changes in microbial carbon processing in the subsoil compared with the topsoil, as is reflected in three aspects. First, the mineralization potential of both SOC (R_{SOC}) and added litter (R_{litter}) decreases in the subsoil (but not topsoil) under the warming and drought relative to ambient treatments (Figure 2), suggesting decreased microbial capacity for carbon mineralization at depth. Second, in line with inhibited microbial mineralization, warming and drought decrease the potential specific activity of certain hydrolyses (in particular, leucine aminopeptidase) in the subsoil, but increase that of phenol oxidase in the topsoil (Figure 3). Third, microbial efficiency for biomass synthesis (CUE′) and necromass accumulation (CAE) decreases relative to respiration in the subsoil under warming and drought (Figure 4d,f). The above changes are observed in all three litter amendments, suggesting field treatment—rather than laboratory incubation—induced decrease of microbial activity and efficiency at soil depth but not the surface.

The divergent microbial responses in the top- versus subsoils may be closely related to (i) the contrasting environments and microbial properties in different soil layers and (ii) declining N availability in the subsoil (but not topsoil) under warming and drought. Subsoil microbes are known to be carbon- or energy-deprived (Jones et al., 2018) and are more responsive to changing labile carbon supply compared with topsoil microbes (Fontaine et al., 2007; Jones et al., 2018). In the studied alpine grassland, subsoil microbes are also N-limited, indicated by the low availability of N relative to labile carbon (i.e., IN/WEOC ratio; Figure 1f; Thomas et al., 2017) and also manifested in the positive correlation of IN/WEOC to the activity of leucine aminopeptidase ($r = 0.39$; $p < 0.05$; Figure S4b) involved

in the release of organic N (Allison et al., 2014). By comparison, the relationship between IN/WEOC and leucine aminopeptidase activity is absent in the topsoil with a relatively higher N availability (i.e., IN/WEOC ratio; Figure 1f; Figure S4a).

Warming and drought treatments at the Haibei Station are shown to increase the relative abundance of deep-rooted Gramineae in the native plant community, thereby enhancing deep root distribution, BNPP at depth (Liu et al., 2018), and labile carbon supply to the subsoil (Figure 1c). According to the microbial economic theory, microbes may minimize their energy costs and decrease extracellular enzyme production when labile carbon is available (Allison & Vitousek, 2005; Bradford, 2013). Hence, elevated root deposition and labile carbon supply may suppress microbial synthesis of hydrolases in the subsoil (Allison & Vitousek, 2005; Wei et al., 2019). Moreover, deep root distribution potentially intensifies plant–microbial competition for nutrients (especially IN) in the subsoil (Hill & Jones, 2019; Jia et al., 2017; Liu et al., 2018), which may further constrain subsoil microbial metabolism and synthesis of extracellular enzymes (Allison & Vitousek, 2005; Schimel & Weintraub, 2003), in addition to the drought stress induced by warming and drought treatments (Burns et al., 2013; Mcdaniel et al., 2013; Sardans & Penuelas, 2005). The response of hydrolases in the subsoils stands in contrast to phenol oxidase activity in the topsoil (Figure 3e), which is more contingent on temperature and oxygen rather than moisture content (Seo et al., 2015; Zuccarini et al., 2020) and thus increases with warming and accompanied aeration under drying (Seo et al., 2015; Sistla & Schimel, 2013).

As a result of the above processes, the warming and drought treatments aggravated microbial N limitation in the subsoil, evidenced by or related to three observations: (i) reduced field concentrations of NO_3^- (Figure 1e) and (ii) decreased ratio of IN/WEOC under warming (Figure 1f); and (iii) reduced potential specific activity of leucine aminopeptidase related to N transformation under drought and warming (Figure 3d). Although NH_4^+ is widely considered to be the preferred form of IN for microbes (Jones & Richards, 1977), a prior incubation study employing ^{15}N-labeling showed that microbial assimilation rate of NO_3^- was higher than that of NH_4^+ at 15°C in soils collected from our experimental site (Wang et al., 2017). This result suggests similar or even stronger potential uptake of NO_3^- relative to NH_4^+ by microbes in this alpine grassland. Hence, the significant decrease of NO_3^- in the warmed and, to a lesser extent, drought-affected subsoil (Figure 1e) evidences a declined absolute availability of IN to microbes, in addition to its reduced abundance relative to labile carbon (WEOC).

The aggravated N limitation and its effect on soil enzyme activities most likely underpin the reduced subsoil microbial mineralization and efficiency under warming and drought (Allison & Vitousek, 2005; Bicharanloo et al., 2020; De Nijs et al., 2019; Li et al., 2019; Manzoni et al., 2012; Schimel & Weintraub, 2003). This conclusion is supported by the strong positive correlations of both R_{SOC} and R_{litter} with leucine aminopeptidase activity in the subsoil (Figure 5; Figure S4b) whereas both microbial CUE′ and CAE are best correlated with the IN/WEOC ratio in the subsoil and across depths (Figure 5i,m; Table S4). Moreover, while CUE′ increases with increasing activity of leucine aminopeptidase in the subsoil, CAE does not (Figure 5). As microbial residues (including amino sugars) are N-enriched organic substrates (Heuck et al., 2015), they may be recycled and/or decomposed by microbes especially under N limitation (Cui et al., 2020; Kaiser et al., 2014). Hence, N availability may have an even stronger control on CAE than CUE′ in the subsoil, as is reflected by the higher *r* value of correlations with IN/WEOC for CAE than CUE′ (Figure 5i,m).

Other than extracellular enzymes, microbial biomass and community structure are also known to regulate microbial mineralization potential and efficiency (Cao et al., 2019). However, based on our PLFA analysis, neither microbial biomass nor community structure (F/B and G+/G−) in the subsoil is affected by field treatments (Figure 4a–c). Moreover, microbial biomass only exerts a secondary effect after the specific activities of phenol oxidase and leucine aminopeptidase on R_{SOC} and R_{litter} across depths (Figure 5). Hence, microbial capacity for carbon mineralization is best explained by extracellular enzyme activities in this study. Nevertheless, we cannot rule out the possibility that nuanced shifts in microbial taxonomic composition and functions (not captured by the PLFA analysis; Zhang et al., 2016) may also contribute to the observed alteration in subsoil microbial carbon processing after warming and drought.

Finally, it is notable that both R_{litter} and microbial CUE′ are higher for the leaf than root amendment in the subsoil but not topsoil (Figures 2b and 4d), while all other microbial and enzyme properties are similar between the two amendments. This result agrees with the generally higher degradability of leaf relative to root litter, reflected in the higher C/N ratio (Table S1) as well as lignin and polyphenol contents of roots relative to leaves (Sun et al., 2018). Microbial CUE′, closely related to R_{litter} (Figure S4b), is hence higher in soils with more degradable litter (Manzoni et al., 2012). The difference in litter decomposability and thus CUE′ is, however, muted in the topsoil with a lower specific activity of extracellular enzymes and a generally lower mineralization potential. Compared with CUE′, CAE seems less sensitive to litter decomposability but more responsive to N limitation (as discussed previously).

5 | CONCLUSIONS

In summary, our study demonstrates that warming and, to a lesser extent, drought in the QTP alpine grassland decreased NO_3^- concentrations and IN/WEOC ratios in the subsoil, intensifying microbial N limitation at depth. Both treatments also decreased microbial capacity of carbon mineralization in the subsoil, likely related to the aggravated N (and moisture) limitation evidenced by lower hydrolase activity (especially leucine aminopeptidase) and reduced microbial efficiency for both biomass synthesis and necromass accumulation. However, none of these effects were observed in the topsoil, suggesting that soil microbes become inactive and inefficient under warming and drought in the alpine grassland subsoil but not topsoil. Given increasing BNPP (Liu et al.,

2018) in the alpine grassland under warming, new inputs of plant-derived carbon may have an elevated sequestration potential in the subsoil, leading to the increased deposits of new carbon in the subsoil without invoking the microbial conversion pathway (Cotrufo et al., 2015). Hence, both elevated root deposits and diminished microbial activity contribute to new carbon accrual observed in the fine-sized fraction of subsoil at this site (Jia et al., 2019). However, decreased hydrolase activity (especially those involved in N and P releases) may slow down the nutrient cycle and further aggravate nutrient limitation in the alpine grassland at depth. Hence, the sustainability of plant growth and persistence of subsoil SOC pools in the long term deserve further investigation in a warmer climate.

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CONFLICT OF INTEREST

The authors have no competing interests that might be perceived to influence the results and discussion reported in this paper.

AUTHOR CONTRIBUTIONS

Xiaojuan Feng and Juan Jia designed the study. Jin-Sheng He, Hao Wang, and Zhenhua Zhang designed and carried out the field experiment. Zhenjiao Cao, Erxiong Zhu, Juan Jia, and Chengzhu Liu carried out sample and data analyses with help from Guohua Dai. Erxiong Zhu, Zhenjiao Cao, and Xiaojuan Feng wrote the paper with inputs from all co-authors.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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