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Effects of short- and long-term nutrient addition on microbial carbon use efficiency and carbon accumulation efficiency in the Tibetan alpine grassland

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ABSTRACT

Microbial activities and physiological performances can directly affect the use and sequestration of organic carbon (C) in soil. Microbial carbon use efficiency (CUE) and carbon accumulation efficiency (CAE) are critical parameters hypothesized to control C transformation and sequestration in terrestrial ecosystem, and may be influenced by nutrient availability. However, knowledge regarding the influence of short- and long-term nutrient addition on microbial CUE and CAE is lacking, particularly in microbial CAE. This study aimed to investigate the effects of nutrient addition on microbial CUE and CAE and the underlying mechanisms. Here, based on a long-term nutrient addition field experiment on the Qinghai-Tibetan Plateau (QTP), we conducted an incubation experiment with and without the ^{13}C-labeled glucose approach to assess microbial CUE and CAE in the top- (0–10 cm) and subsoil (20–30 cm) collected in the second (short-term) versus tenth (long-term) fertilized year. According to the microbial economic theory, short-term nitrogen (N) addition suppressed oxidase activities in the topsoil, whereas long-term nutrient addition significantly changed hydrolase activities. Furthermore, the short- and long-term nutrient additions had no significant effect on microbial CUE, which may be attributed to the fact that the slightly increased content of C allocated to microbial biomass production was not sufficient to significantly change the CUE. Microbial CAE only increased with 10-year of continuous N addition, which was mainly due to increasing N availability. We found that the C-acquisition enzymes and clay content dominantly regulated microbial CUE, whereas N availability played an important role in regulating CAE. In conclusion, our results elucidated the differential responses of microbial CUE and CAE to N and phosphorus (P) addition, providing empirical evidence for understanding microbially governed C and their feedback to nutrient interactions in the context of increasing anthropogenic N and P input.

1. Introduction

As decomposers and contributors to soil organic carbon (SOC), microbes are not only involved in the decomposition of SOC by secreting extracellular enzymes (Bond-Lamberty et al., 2018) but also assimilate plant-derived carbon (C) into microbial biomass and finally accumulate in soils as microbial-derived C (i.e., living biomass, extracellular compounds, and necromass), contributing to a stable SOC pool (Cotrufo et al., 2013; Liang et al., 2016; Zheng et al., 2021; Zhu et al., 2020). Global nitrogen (N) deposition and phosphorus (P) inputs have been increasing because of increasing fossil fuel use and intensive fertilizer application (Galloway et al., 2008; Yuan et al., 2018), and have a tremendous impact on plant growth (Fay et al., 2015), microbial metabolism (extracellular enzyme activities and functions), and community composition (Fang et al., 2019; Widdig et al., 2020a), which in turn influences the transformation and accumulation of SOC (Yuan et al., 2021). Microbial C use efficiency (CUE) and C accumulation efficiency (CAE) are critical parameters controlling soil C degradation and

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sequestration. Microbial CUE, which is defined as the ratio of C allocated to microbial growth to total C uptake, was used to evaluated the amount of C taken up by microbes and incorporated into the microbial biomass (Geyer et al., 2019; Manzoni et al., 2012; Sinsabaugh et al., 2013). CAE is defined as microbial necromass (indicated by amino sugar) synthesized from "new" C relative to metabolized "new" C and is used to evaluate the efficiency of microbes in converting degradable C into necromass (Jia et al., 2017; Zhu et al., 2021). Hence, evaluating the effects of increasing N and P enrichment on CUE and CAE and their governing factors may provide a perspective for understanding microbial responses to changes in the environment.

As a critical metric for balancing microbial catabolism and anabolism (Schimel and Schaeffer, 2012), several studies have demonstrated that CUE can be affected by C substrate availability, soil properties, and extracellular enzyme activities (Frey et al., 2013; Manzoni et al., 2012; Sinsabaugh et al., 2013). Previous studies have indicated various responses of CUE to short-term (< 5 years) N and P addition in different ecosystems, including enhancement (Luo et al., 2020a), reduction (Riggs et al., 2015), and lack of significance (Lee and Schmidt, 2014). Moreover, long-term (> 5 years) N and P addition had different effects on microbial CUE. Spohn et al. (2016) found increased CUE with 54-year N and P addition, which was mainly due to suppressed activities of oxidative enzymes involved in the decomposition of complex compounds and the reduction of energy requirement for N by microbes. Nevertheless, a decreased CUE was also found in a temperate grassland in a 6-year N addition experiment, which was induced by a decreased ratio of fungi to bacteria (Riggs and Hobbie, 2016). Widdig et al. (2020b) reported an unchanged CUE with > 7-year N and P addition and attributed this to the same direction and magnitude of the effects on microbial growth and respiration. In addition, clay affects microbial CUE by altering the substrate availability (Islam et al., 2023). As the amount of clay increases, the availability of substrate to microbes is reduced owing to the absorption and protection of C substrate (Pei et al., 2021; Sokol et al., 2019), which may lead to a lower microbial CUE. In summary, studies focusing on the effects of short- or long-term nutrient addition on microbial CUE have shown with inconsistent results (Feng et al., 2022). Studies on the responses of microbial CUE to short- and long-term nutrient addition in the same field experiment may shed light on adequate understanding of the effects of fertilization duration on microbial C processes.

Compared with the effects of nutrient addition on CUE, less is known about CAE (Cai et al., 2022). There is increasing evidence that microbial necromass contributes to stable SOC (Liang et al., 2017; Ma et al., 2018a), and assessing the ratio of microbial production of amino sugars relative to respiration is thus crucial for evaluating microbial SOC accumulation (Cai et al., 2022; Jia et al., 2017; Zhu et al., 2021). Some studies have analyzed the response of microbial necromass to N and P addition by altering microbial growth and activities (Hu et al., 2022; Liang and Balser, 2012; Luo et al., 2022; Ma et al., 2018b; Yuan et al., 2021). For example, the suppressed activity of residue-decomposing enzymes has led to increased microbial necromass with 10-year P addition in a subalpine forest (Luo et al., 2022), whereas the enhanced activity of residue-decomposing enzymes led to decreased microbial necromass with 10-year P addition in a tropical coastal forest (Yuan et al., 2021). Liang and Balser (2012) suggested that microbial necromass slightly decreased with 9-year N addition in a Mediterranean grassland, which can be attributed to the decline in arbuscular mycorrhizal fungi. In addition, microbial CAE may be potentially influenced by clay. The clay content may contribute to the accumulation of microbial necromass owing to mineral protection (Cai et al., 2022; Ma et al., 2018a; Six et al., 2006). Overall, different ecosystems with different soil properties (Ma et al., 2018b) and microbial community properties and activities (Liang and Balser, 2012; Luo et al., 2022) may have different effects on microbial CAE with N and P additions. However, only the content and accumulation mechanisms of microbial necromass have been investigated (Hu et al., 2022; Ma et al., 2018a; B.R. Wang et al., 2022) and studies on CAE and its regulatory mechanisms remain poorly understood. In particular, it remains unknown whether and how microbial CAE respond to short- and long-term nutrient addition.

The QTP is sensitive to climate change and human activities (S.B. Liu et al., 2018; Wang et al., 2012), and has also been experiencing increased N deposition and P input (Zhu et al., 2016). The QTP stores 7.4 Pg C in the 0–10 cm depth soil (Yang et al., 2008), and minor shifts in SOC induced by N and P deposition could have a dramatic influence on SOC dynamics in terrestrial ecosystems (Chen et al., 2013). Here, based on a long-term N and P addition field experiment (since 2011) in the alpine grasslands of the QTP. We performed a 45-day incubation experiment with and without ^{13}C-labeled glucose using the topsoil (0–10 cm) and subsoil (20–30 cm) collected in the second (short-term, 2012) and tenth years (long-term, 2020). Microbial CUE and CAE were assessed using microbial biomass carbon (MBC) and amino sugars, respectively, combined with ^{13}C analysis. Coupled with soil properties, plant biomass, and microbial enzyme activities, this study aimed to evaluate the effects of short- and long-term N and P addition on microbial CUE and CAE in the topsoil versus subsoil, and to elucidate the underlying mechanisms driving microbial activities and functions in response to different durations of fertilization in alpine grasslands.

Specifically, we hypothesized that (1) both microbial CUE and CAE will increase with short-and long-term nutrient addition due to mitigated N and P limitations (Y.F. Wang et al., 2022; Zhu et al., 2021), and (2) microbial CUE will be controlled by microbial biomass and activities (Spohn et al., 2016), whereas CAE is controlled by clay content (Cai et al., 2022).

2. Materials and methods

2.1. Site description and experimental design

The field experiments on long-term nutrient addition were conducted at the Haibei Alpine Grassland Ecosystem Research Station (37° 29′−37°45′N, 101° 12′−101° 23′E, 3200 m above sea level) located in the northeast of the Tibetan Plateau in Qinghai Province, China. The region has a typical monsoon climate, with a long, cold winter and a short, cool summer. The mean annual temperature and precipitation were −1.2 °C and 489 mm, respectively, from 1980 to 2014. Approximately 80 % of precipitation occurs from May to September during the growing season (Fang et al., 2014). The soil in the study was classified as Cambisol (IUSS Working Group WRB, 2015). The dominant native plant species are *Kobresia humilis*, *Stipa capillata*, *Elymus nutans*, *Gentiana straminea,* and *Festuca ovina* (Luo et al., 2020b).

The N and P addition experiment was initiated in May 2011 by fencing off an area of six blocks (20 m × 60 m) to prevent grazing distribution. Each randomized complete block included six treatments: CK (with no nutrient addition), N addition (N1, 25 kg N ha^{-1} yr^{-1} as urea; N2, 50 kg N ha^{-1} yr^{-1} as urea; N3, 100 kg N ha^{-1} yr^{-1} as urea), P addition (50 kg P ha^{-1} yr^{-1} as triple superphosphate), and N + P addition (100 kg N ha^{-1} yr^{-1} as urea and 50 kg P ha^{-1} yr-^{1} as triple superphosphate). The background N deposition ranged from 1.0 g N m^{-2} year^{-1} to 1.5 g N m^{-2} year^{-1} at the experimental site in the early 20th century (Jing et al., 2016), whereas N addition in the study was substantially higher than atmospheric N deposition. The field experiment consisted of 36 plots, including six replicate plots (6 m × 6 m). Each block and plot was separated by 2-m-wide and 1-m-wide buffer walkways to prevent distribution from neighboring plots. The N fertilizer in the form of urea and P fertilizer in the form of triple superphosphate were distributed into three equal parts and evenly spread by hand onto the ground surface at the beginning of June, July, and August during the growing season each year. The fertilizers were applied after sunset or before rainfall at high moisture levels and low temperatures to avoid solubilization loss and leaching.

2.2. Plant and soil sampling

On 28 August 2012 and 24 August 2020, topsoil (0–10 cm) and subsoil (20–30 cm) samples were collected using a 5-m diameter soil corer from four selected treatments of the above six treatments: CK (with no nutrient addition), N addition (N3, 100 kg N ha^{-1} yr^{-1} as urea), P addition (50 kg P ha^{-1} yr^{-1} as triple superphosphate), and N + P addition (100 kg N ha^{-1} yr^{-1} as urea and 50 kg P ha^{-1} yr^{-1} as triple superphosphate). Three replicates were randomly selected from six corresponding blocks, and three soil cores were taken from the same plot and thoroughly mixed in situ at each depth to obtain a single composite sample. The soil samples were packed in a constant-temperature box with ice and immediately taken to the laboratory. The samples were then passed through a 2-mm sieve to remove visible vegetable debris and stones. After sieving, the samples collected in 2012 were kept at − 80 °C until 2020. The samples collected in 2020 were transported to the laboratory and sieved. Meanwhile, the soil samples collected in 2012 were removed from − 80 °C and thawed at 4 °C. Subsequently, part of the soil sample from both years (2012 and 2020) was kept at 4 °C for incubation experiments and analysis of extracellular enzyme activities, whereas the part was freeze-dried for analysis of soil properties. Plant aboveground biomass (AGB) was measured annually by clipping within a 25 cm × 25 cm quadrant in each 6 m × 6 m plot. The root biomass was collected from three replicate cores in each plot using a 5 cm-diameter soil auger, which was used to measure belowground biomass (BGB). The plant and root samples were taken to the laboratory and cleaned by immersion in deionized water. The AGB and BGB were determined after oven-drying the plant tissues to a constant mass at 65 °C for 48 h.

2.3. Soil incubation experiment

A 45-day incubation experiment was conducted to ascertain the microbial CUE and CAE. Approximately 20 g of soil stored at 4 °C was weighed into a 165 ml brown culture flask in an incubator and maintained at 65 % water-holding capacity by spraying Milli-Q water on the soil. Before incubation, the samples were pre-incubated at 25 °C for one week to activate soil microbes. Afterwards, soils were divided evenly into two parts: one identified as a control group without any amendment and the other as a ^{13}C-labeled glucose group. The ^{13}C-labeled glucose (Sigma-Aldrich, uniformly labeled, 2 atoms %) diluted with Milli-Q water was splashed evenly onto the soil, and the same amount of Milli-Q water was added to the control group. The amount of added glucose was equivalent to 2 % of SOC in the corresponding soil sample to fully alleviate carbon limitation to soil microbes and trace the added labile carbon incorporated into microbial biomass and residues in a relatively short period (Jia et al., 2017).

At hour 2 and 10, as well as days 1, 3, 6, 10, 15, 20, 30, and 45 of incubation, soil respiration was quantified using a gas chromatography machine (GC; Agilent 7890a, USA), sealed, and incubated for 6 h before being filled with air without CO_2 in the bottle. An isotope ratio mass spectrometer (IRMS; Delta PLUS XP, Thermo Finnigan, Germany) was used to measure the δ^{13}C values of the respired CO_2 (six times) from glucose to distinguish between CO_2 derived from SOC and glucose. On Day 45, incubation was terminated when the soil respiration rates of the ^{13}C-labeled glucose group were comparable to those of the control group. At the end of the incubation experiment, the soils were subjected to destructive sampling and a series of subsequent measurements (Sections 2.4 to 2.7).

2.4. Soil properties analysis

The SOC and total nitrogen (TN) content were determined using an element analyzer (Vario EL III, Elementar, Hanau, Germany) after removing inorganic C with hydrochloric acid (HCl). Total phosphorus (TP) was extracted by digestion with H_2SO_4-$HClO_4$ and examined using an Auto Discrete chemical analyzer (AMS Smarchem450, Italy). Soil inorganic nitrogen (IN) was extracted using a 1:10 (w:v) soil-to-solution ratio of 2 M potassium chloride solution and then quantified using an Auto Discrete chemical analyzer (Smart Chem 450, AMS, Italy). The ratio of IN to DOC (expressed as IN/DOC hereafter) was used to evaluate the availability of IN relative to the available OC in the soil. The soil pH was measured with a pH meter after shaking the soil-to-suspension of 1:2.5 (w:v) for 30 min and suspending it for 6 h. The MBC was examined using the chloroform fumigation-extraction method (Vance et al., 1987). Fumigated and non-fumigated soil samples were extracted with 0.5 M K_2SO_4 in a soil solution with a soil-to-solution ratio of 1: 5 (w:v). A total organic carbon analyzer (Multi N/C 3100, Analytik Jena, Germany) was used to determine the filtered liquor. The OC content of the non-fumigated samples was defined as the dissolved organic carbon (DOC) content. MBC was calculated as the OC difference between non-fumigated and fumigated samples divided by a factor of 0.45. Soil texture was measured using a Malvern Mastersizer 2000 particle analyzer (Ryzak and Bieganowski, 2011).

2.5. Enzyme analysis

At the end of the incubation, we measured five major hydrolases, including α-glucosidase (AG), β-1,4-glucosidase (BG), alkaline phosphatase (AP), L-leucine-*p*-aminopeptidase (LAP), and β-1,4-*N*-acetylglucosaminidase (NAG) and one major oxidase (phenol oxidase) in fresh soil from destructive sampling to explore the extracellular enzyme activity of soils. Briefly, 1 g of fresh soil was mixed with 125 ml of Milli-Q water and homogenized using a magnetic stirrer for 10 min. For hydrolases, the sample suspension (200 μL) was piped into black 96-well microplates with 50 μL of 200 μM 4-methylumbelliferone (MUB) (for AG, BG, AP, and NAG) and 7-amino-4-methylcoumarin (MUC) (for LAP) dissolved in sodium acetate buffer solution (pH = 5) and incubated at 25 °C for 4 h in the dark. Each sample was analyzed in six replicate wells. For each soil sample, standard curves of MUB and MUC with eight concentration gradients were measured. For phenol oxidase, the sample suspension (200 μL) was piped into transparent 96-well microplates with 50 μL of 200 μM L-3,4-dihydroxyphenylalanine (L-DOPA) dissolved in Tris buffer (pH = 8.2) and incubated in the dark for 3 h at 25 °C. Each sample was analyzed in eight replicate wells. After incubation, the fluorescence of hydrolases was measured by excitation at 365 nm and emission at 450 nm, and the absorbance of oxidases was measured at 450 nm using a Microplate Reader (Thermo Fisher Scientific, USA). The extracellular enzyme activities were expressed as μmol g^{-1} SOC h^{-1} or mmol g^{-1} SOC h^{-1}.

2.6. The δ^{13}C-MBC and CUE

To differentiate microbial biomass synthesized from ^{13}C-labeled glucose, the δ^{13}C values of MBC were calculated. The remaining K_2SO_4 extraction from MBC was freeze-dried, and the δ^{13}C values of the extracted C were determined using stable isotope mass spectrometry (EAIRMS, Isoprime, England). The δ^{13}C value of the MBC was calculated using the following equation:

$$\delta^{13}C_{MBC} = \frac{\delta^{13}C_{fumi} \times C_{fumi} - \delta^{13}C_{non-fumi} \times C_{non-fumi}}{C_{fumi} - C_{non-fumi}} \times 100\% \quad (1)$$

where $\delta^{13}C_{fumi}$ and $\delta^{13}C_{non\text{-}fumi}$ represent the δ^{13}C values of the fumigated and non-fumigated soils, respectively, and C_{fumi} and $C_{non\text{-}fumi}$ represent the DOC of the fumigated and non-fumigated soils, respectively.

MBC derived from glucose was calculated using the following equation (Zhu et al., 2021):

$$MBC_{glucose} = MBC_{total} \times \frac{\delta^{13}C_{MBC} - \delta^{13}C_{MBC-UN}}{\delta^{13}C_{glucose} - \delta^{13}C_{MBC-UN}} \times 100\% \quad (2)$$

where $\delta^{13}C_{MBC}$ and $\delta^{13}C_{MBC\text{-}UN}$ represent the δ^{13}C values of MBC in the glucose-amended and glucose-unamended soils, respectively. $\delta^{13}C_{glucose}$ represents the $\delta^{13}C$ value of the glucose amendment.

Microbial CUE was calculated based on MBC (Bradford et al., 2013) as follows:

$$CUE = \frac{MBC_{glucose}}{MBC_{glucose} + CO_{2C-glucose}} \times 100\% \quad (3)$$

Where $MBC_{glucose}$ represents the C content of MBC derived from glucose after the incubation experiment. $CO_{2\ C\text{-}glucose}$ represents the C content released from respiration derived from glucose.

For microbial physiological traits, the C_{growth}, $C_{respiration}$, C_{uptake}, and turnover time of microbial biomass were calculated using the following equations (Spohn et al., 2016):

$$C_{growth} = MBC_{glucose} \quad (4)$$

$$C_{respiration} = CO_{2C-glucose} \quad (5)$$

$$C_{uptake} = C_{growth} + C_{respiration} \quad (6)$$

$$\text{Turnover time} = \frac{MBC}{C_{growth}} \quad (7)$$

where C_{growth} represents the content of C allocated to microbial biomass production, $C_{respiration}$ represents the content of C allocated to CO_2 (respiration), C_{uptake} represents the content of C uptake by microbial biomass, and MBC represents microbial biomass carbon.

2.7. Amino sugar and CAE

Amino sugars were used to determine the quantity of microbial necromass according to Zhang and Amelung (1996). Myo-inositol and methylglucamine were considered internal and recovery standard, respectively. A gas chromatograph equipped with a quadrupole mass spectrometer (GC-MS; Shimadzu QP2020, Japan) was used to quantify the amino sugar compounds. The details are documented in the Supplementary methods.

To distinguish microbial necromass synthesized from glucose versus SOC, a stable isotope ratio mass spectrometer (Thermo MAT 253, Germany) via a combustion interface (GC-C-IRMS) using conditions similar to those used in GC/MS analysis was used to measure the $\delta^{13}C$ isotopic composition of the two main amino sugars with sufficient abundance in soils (glucosamine and galactosamine). A mass-balancing method was used to correct the $\delta^{13}C$ values of the amino sugars for the glucose-derived carbon added to the amino sugars (Cao et al., 2019; Jia et al., 2017). Glucosamine and galactosamine serve as the foundation for abundance-weighted average $\delta^{13}C$ amino sugars.

The proportion of glucose-derived C in amino sugars was calculated as follows:

$$f_{glucose} = \frac{\delta^{13}C_C - \delta^{13}C_{SOC}}{\delta^{13}C_{glucose} - \delta^{13}C_{SOC}} \times 100\% \quad (8)$$

where $f_{glucose}$ represents the percentage of compounds derived from ^{13}C-labeled glucose and $\delta^{13}C_{glucose}$, $\delta^{13}C_C$, and $\delta^{13}C_{SOC}$ represent the abundance-weighted average $\delta^{13}C$ for the added glucose, target compounds (glucosamine and galactosamine), and SOC, respectively.

Microbial CAE, which was used to evaluate the efficiency of microbes in transforming decomposable C into necromass, was calculated as follows (Jia et al., 2017):

$$CAE = \frac{\text{Amino sugar}_{C-glucose}}{\text{Amino sugar}_{C-glucose} + CO_{2C-glucose}} \times 100\% \quad (9)$$

where amino $sugar_{C\text{-}glucose}$ is the amount of amino sugar synthesized with glucose, calculated by multiplying $f_{glucose}$ by the amino sugar concentration. $CO_{2\ C\text{-}glucose}$ represents the C content released from respiration derived from glucose.

2.8. Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics 25 (IBM SPSS Statistics 25, Chicago, IL) or R soft (version 4.1.3). Shapiro-Wilk was used to examine the normal distribution of data. If normality was not attained, non-parametric tests (Mann-Whitney U-test) and post-hoc analysis (Kruskal-Wallis H test) were performed. The differences in microbial enzyme activities were examined using a three-way analysis of variance (ANOVA) with treatments, soil depths, and glucose amendment as independent variables. The differences in microbial enzyme activities, CUE, and CAE were examined using two-way analysis of variance (ANOVA) with treatments and soil depth as independent variables. The data on soil properties, plant biomass, enzyme activities, and CUE were *log*-transformed to achieve a normal distribution (checked using the Shapiro-Wilk test). Redundancy analysis (RDA) was conducted to visualize the correlations among microbial CUE, CAE, soil properties, plant biomass and enzyme activities using "vegan" package in R software (version 4.1.3). The relationships between environmental variables (including soil properties, plant biomass, and enzyme activities) and microbial CUE were examined using Pearson linear correlation, and those using CAE were examined with Spearman linear correlation, as the data cannot be normally distributed. Environmental variables exhibiting significant correlations with the examined parameters were selected using multiple stepwise regression (stepping method) to further clarify the factors dominating the microbial mineralization potential, CUE, and CAE. The correlations and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Extracellular enzyme activity

After short-term nutrient addition, SOC-normalized specific hydrolase activities (including AG, BG, AP, and NAG) were higher in the topsoil, whereas PO activities were higher in the subsoil ($p < 0.05$; Fig. 1). The hydrolase activities did not significantly change with different treatments ($p > 0.05$; Fig. 1). However, the activities of PO significantly decreased with N addition to the topsoil and increased with P and N + P addition to the subsoil ($p < 0.05$; Fig. 1f).

After long-term nutrient addition, AG, BG, AP, and NAG activities were higher in the topsoil, whereas LAP activities were higher in the subsoil ($p < 0.05$; Fig. 2). In contrast to the short-term nutrient addition, hydrolase activities were significantly changed, whereas PO activities did not change ($p > 0.05$; Fig. 2f). Specifically, long-term N addition increased topsoil AG, AP, and NAG activities; P addition increased subsoil AP and LAP activities but decreased topsoil NAG activities; and N + P addition increased topsoil BG and AP activities ($p < 0.05$; Fig. 2).

3.2. Microbial physiological traits and efficiency

After short-term nutrient addition, C_{growth} (42.43 ± 5.45 μg g^{-1} soil), $C_{respiration}$ (130.6 ± 11.40 μg g^{-1} soil), C_{uptake} (173.0 ± 13.34 μg g^{-1} soil) and turnover time (40.71 ± 5.25 days) in the topsoil was on average 2.13, 0.86, 1.07, 0.25 times higher than that of subsoil, respectively ($p < 0.05$; Fig. 3c, e, g, i). C_{growth} increased significantly with P and N + P addition to the topsoil by 77.7 % and 53.7 %, respectively ($p < 0.05$; Fig. 3c), and the turnover time increased remarkably by 91.3 % with N addition to the topsoil ($p < 0.05$; Fig. 3i). However, microbial CUE (20.69 ± 2.05 %) was not significantly different among different treatments and soil depths ($p > 0.05$; Fig. 3a). In addition, the microbial CAE (0.97 ± 0.25 %) was not significantly different among the different treatments and soil depths ($p > 0.05$; Fig. 4a).

After long-term nutrient addition, C_{growth} (79.06 ± 27.40 μg g^{-1}

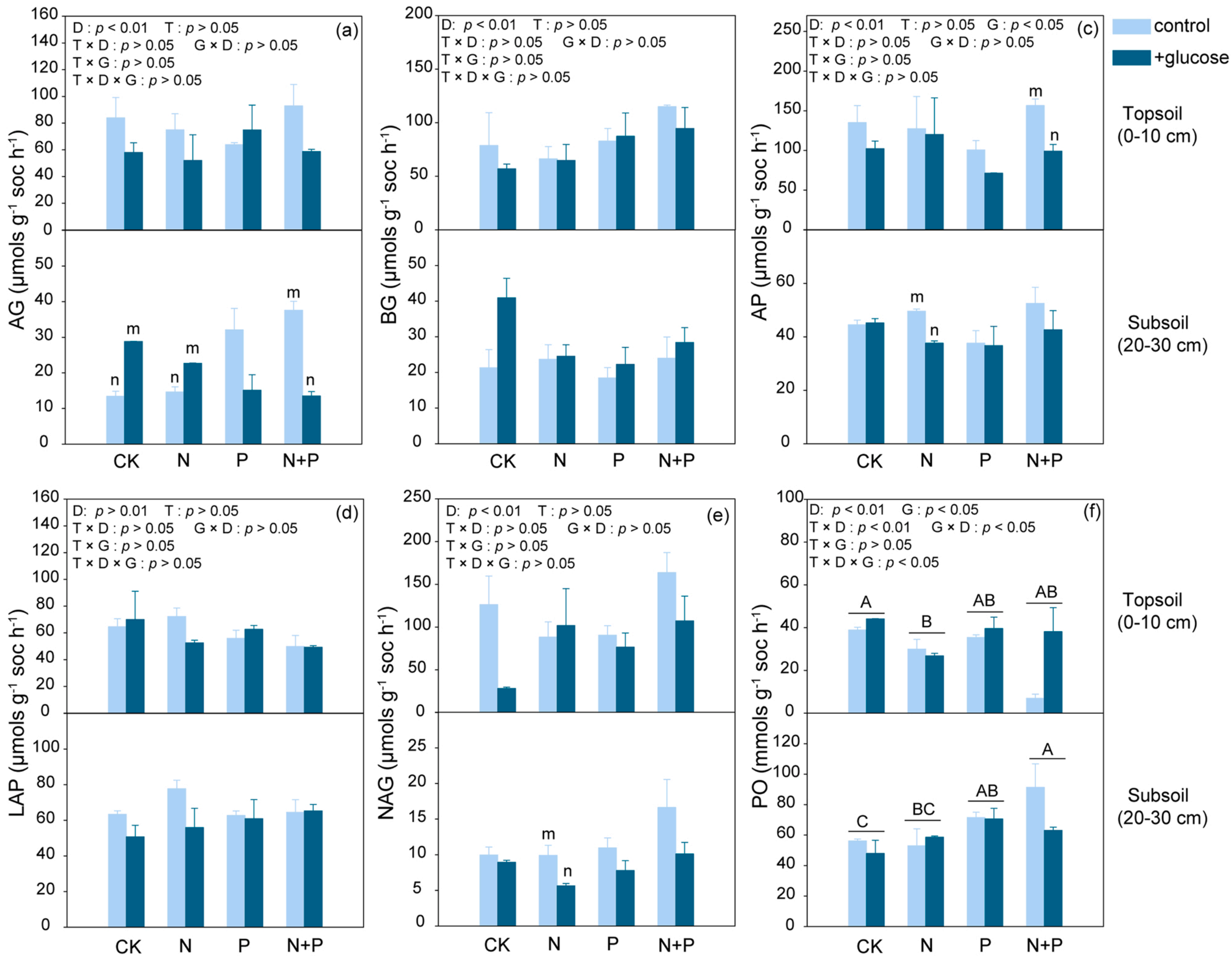

Fig. 1. Effect of short-term nutrient addition on extracellular enzyme activities after the incubation experiment, including AG, α-1,4-glucosidas (a); BG, β-1,4-glucosidase (b); AP, acid phosphatase (c); LAP, leucine amino peptidase (d); N-acetyl-β-D-glucosidase (e); PO, polyphenol oxidase (f). The mean values are shown with standard errors (n = 3). T, D, and G denote the effects of the treatments, depths, and glucose amendment, respectively. Different lowercase letters indicate significant differences between the control and glucose amendment ($p < 0.05$). Different uppercase letters indicate significant differences among the treatments ($p < 0.05$).

soil), $C_{respiration}$ (39.62 ± 11.01 μg g^{-1} soil), and C_{uptake} (120.4 ± 31.99 μg g^{-1} soil) in the topsoil were on average 3.18, 0.74, 1.86 times higher than that of the subsoil, respectively ($p < 0.05$; Fig. 3d, f, h, j), but the turnover time exhibited similar values between the topsoil (14.77 ± 2.46 Days) and subsoil (9.59 ± 1.20 Days; $p > 0.05$; Fig. 3j), and no obvious differences in the above microbial physiological traits were found among the different treatments ($p > 0.05$; Fig. 4). As a result, microbial CUE (63.17 ± 4.70 %) in the topsoil was higher than that in subsoil (45.98 ± 4.39 %) ($p < 0.05$; Fig. 3b), but no significant difference was found among different treatments ($p > 0.05$; Fig. 3b). For microbial CAE, there was no significant change in microbial CAE between the topsoil (2.24 ± 0.28 %) and subsoil (1.89 ± 0.24 %) ($p > 0.05$; Fig. 4b). The N addition increased microbial CAE in the topsoil ($p < 0.05$; Fig. 4b), but no obvious difference was found in CAE among all treatments in the subsoil ($p > 0.05$; Fig. 4b).

3.3. Factors affecting microbial CUE and CAE

RDA was performed to elucidate the factors affecting the microbial CUE and CAE. Our results showed that the first two axis components explained 79.49 % and 84.29 % of the variance for short- and long-term nutrient addition, respectively, indicating the important role of soil properties, plant biomass, and enzyme activities in determining microbial CUE and CAE (Figs. 5 and 6).

To determine the specific factors affecting microbial CUE and CAE, their relationships with soil properties, plant biomass, and enzyme activities were examined using a simple linear correlation (Fig. S1). After short-term nutrient addition, microbial CUE was positively correlated with TP, AG, BG, and sand content but negatively correlated with clay content, and microbial CAE was negatively correlated with TP (Fig. S1). After long-term nutrient addition, microbial CUE was positively correlated with SOC, TN, MBC, DOC, IN, AG, BG, AP, and NAG, whereas microbial CAE was positively correlated with IN (Tab. S2). Moreover, multiple stepwise regression was used to determine the dominant factors affecting the CUE (Table 1). The results showed a significant role for clay content (short-term nutrient addition) and AG (short- and long-term nutrient addition) in microbial CUE (Table 1).

4. Discussion

4.1. Responses of extracellular enzyme activities to nutrient addition

In our study, extracellular enzyme activities responded differently to short- and long-term nutrient addition. After short-term nutrient addition, the activities of PO changed significantly in the top-and subsoil, whereas no significant change was found on hydrolase activities (Fig. 1).

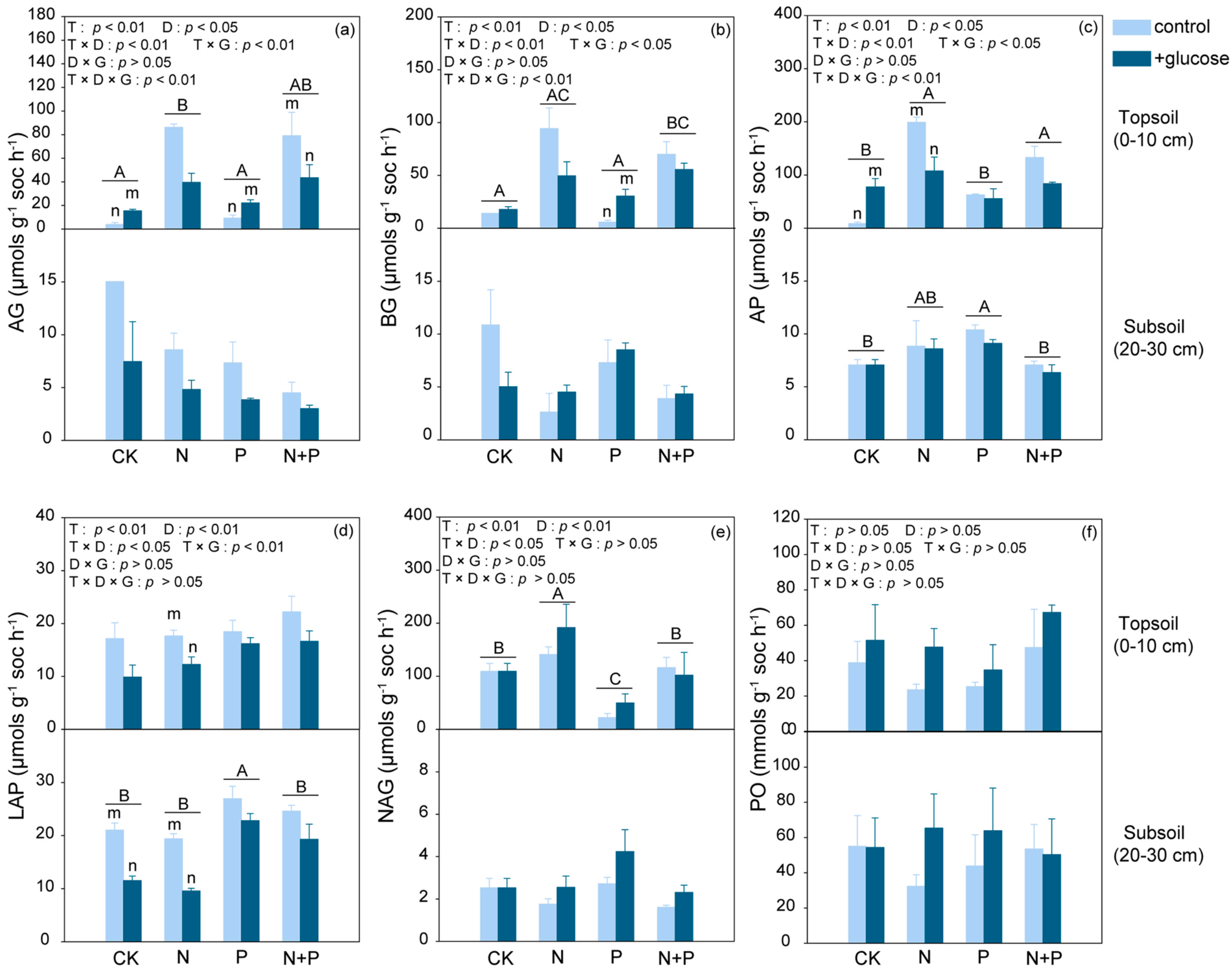

Fig. 2. Effect of long-term nutrient addition on extracellular enzyme activities after the incubation experiment, including AG, α-1,4-glucosidase (a); BG, β-1,4-glucosidase (b); AP, acid phosphatase (c); LAP, leucine amino peptidase (d); N-acetyl-β-D-glucosidase (e); PO, polyphenol oxidase (f). The mean values are shown with standard errors (n = 3). T, D, and G denote the effects of the treatments, depths, and glucose amendment, respectively. Different lowercase letters indicate significant differences between the control and glucose amendment ($p < 0.05$). Different uppercase letters indicate significant differences among the treatments ($p < 0.05$).

Consistent with previous studies (Jing et al., 2017; Ma et al., 2014), short-term nutrient addition did not change the hydrolase activities. This may have been caused by the disadvantaged microbes in the competition for resources, and soil nutrients were preferentially utilized by plants (Liu et al., 2007; Stark and Kytöviita, 2006). This is consistent with the fact that extracellular enzyme activities are regulated more by plant species than nutrient availability in a 40-day incubation experiment of upland grassland topsoil with N addition (Bardgett et al., 1999), Consequently, hydrolase activities were less responsive to short-term nutrient addition.

In contrast to hydrolases, oxidase (i.e., PO) is sensitive to short-term nutrient addition because the activation energy of PO (32.5 kj mol^{-1}) is lower than that of hydrolases (e.g., 61.8KJ mol^{-1} for BG) (Davidson et al., 2012; Hobbie et al., 2012). In our study, the activities of PO decreased with N addition in the topsoil but increased with P and N + P addition in the subsoil (Fig. 1f). PO is mainly secreted by fungi and can decompose phenolic compounds, such as lignin, to obtain nutrients and energy (Datta et al., 2017; Hendel et al., 2005). Short-term N addition decreased the activity of PO in the topsoil owing to the enhanced plant inputs with N addition (N and N + P addition; Tab. S1). According to microbial economic theory, when labile nutrients are abundant, microbes preferentially decompose labile C and inhibit the degradation of recalcitrant C, such as lignin (Allison and Vitousek, 2005; Sinsabaugh et al., 1993), which minimizes their energy costs and reduces the synthesis of PO. However, PO activity tended to increase to acquire more C with P fertilization (P and N + P addition), as P fertilization promoted microbial demand for C (Luo et al., 2019). Based on the contrasting effects of N and P addition on PO activity, the activity did not change in the topsoil with the addition of N + P, but decreased with N addition (Fig. 1f). Moreover, consistent with previous studies, Chen et al. (2018) claimed that the relative abundance of genes encoding PO decreased with N loading, which may have also suppressed PO activity. Similar to that in the topsoil, PO activity increased with short-term P and N + P addition in the subsoil because of the increased microbial demand for C with P addition.

After long-term nutrient addition, based on the resource allocation theory, microbes can regulate the production of different enzymes (Allison and Vitousek, 2005; Sinsabaugh and Moorhead, 1994). In our study, N and N + P addition significantly increased the activities of C-acquisition (AG with N addition and BG with N + P addition in the topsoil) and P-acquisition (AP with N and N + P addition in the topsoil) enzymes ($p < 0.05$; Fig. 2). These results agree with those of previous meta-analyses assays (Jian et al., 2016), and support the theory that microbes follow a survival strategy that would suppress enzyme activities when inorganic nutrients and labile C are available, whereas it increases related enzyme activities with limited resources (Allison and

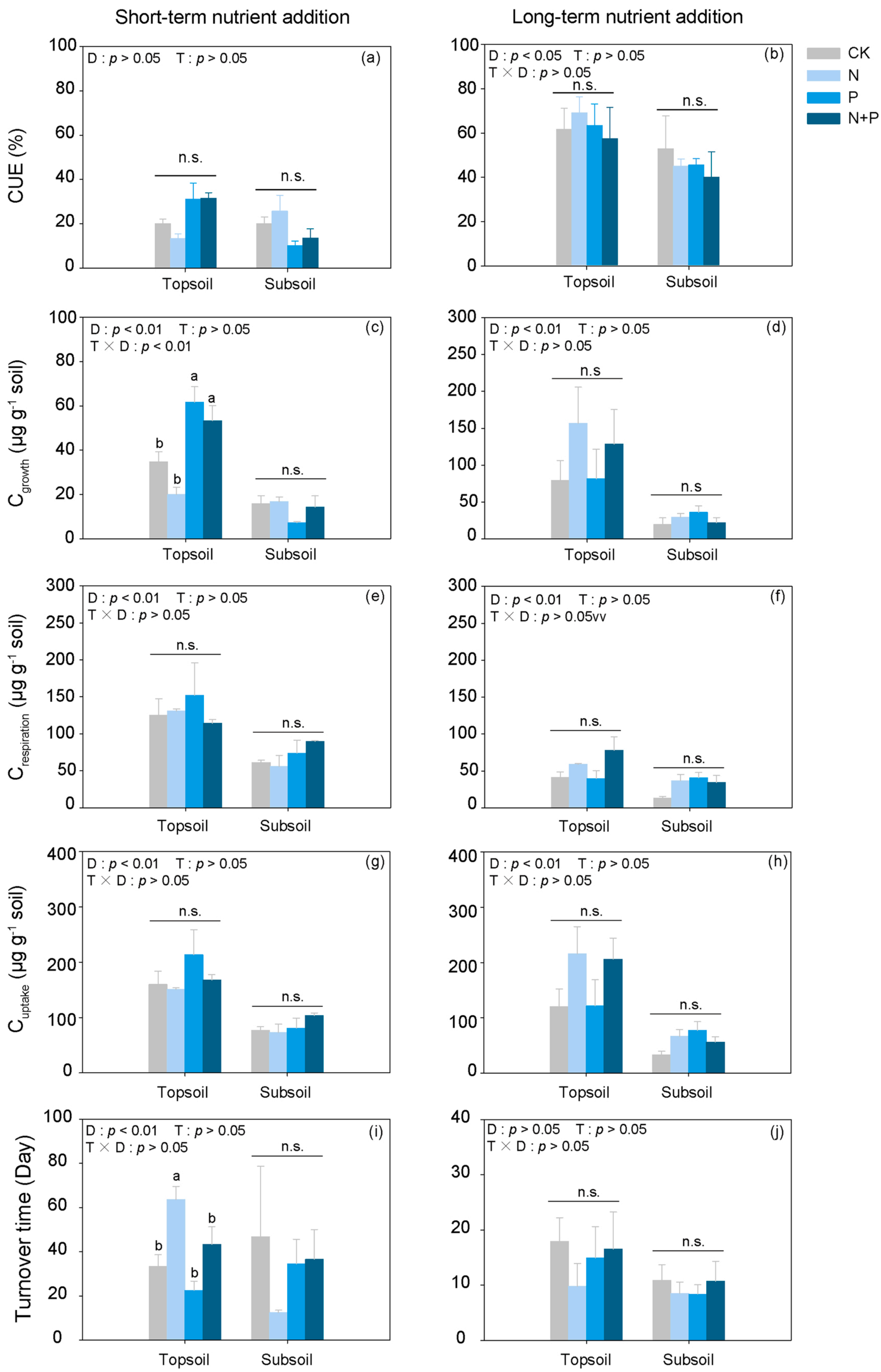

Fig. 3. Effect of nutrient addition on microbial growth traits, including CUE with short-term (a) and long-term nutrient addition (b); C_{growth} with short- (c) and long-term nutrient addition (d); $C_{respiration}$ with short- (e) and long-term nutrient addition (f); C_{uptake} with short- (g) and long-term nutrient addition (h); and turnover time with short- (i) and long-term nutrient addition (g). Mean values are shown with standard errors (n = 3). T and D denote the effects of treatments and depths, respectively. Different lowercase letters indicate significant differences among the treatments ($p < 0.05$). n.s., not significant ($p > 0.05$).

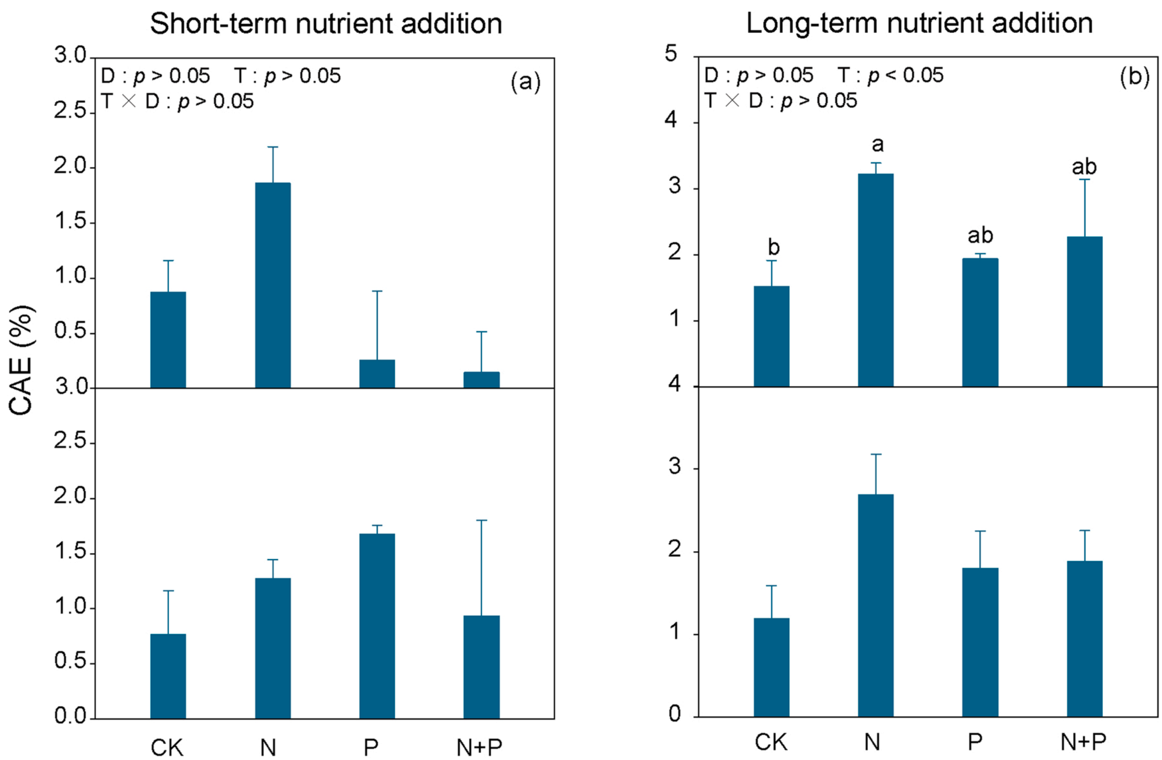

Fig. 4. Effect of nutrient addition on CAE with short- (a) and long-term (b) nutrient addition. Mean values are shown with standard errors (n = 3). T and D denote the effects of treatments and depths, respectively. Different lowercase letters indicate significant differences among the treatments ($p < 0.05$).

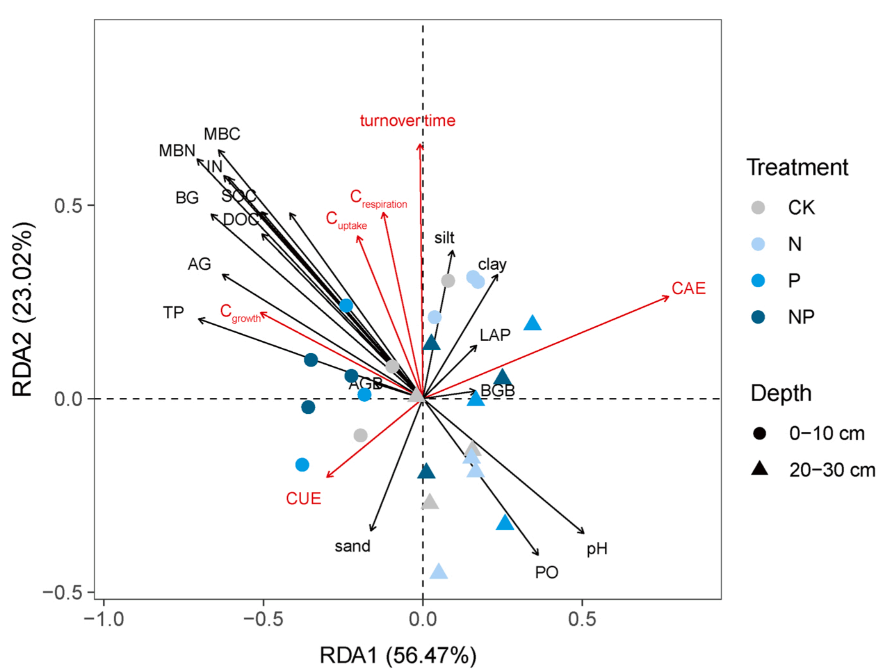

Fig. 5. Redundancy analyses (RDA) for the relationships between soil properties, plant biomass, and enzyme activities (black lines) and microbial physiological traits and efficiency (including CUE, CAE, and C_{growth}, $C_{respiration}$, C_{uptake}, and turnover time) (red lines) in the top- and subsoil with short-term nutrient addition. The different colors of the points indicate four treatments, and the different shapes indicate two depths.

Vitousek, 2005; Miao et al., 2019; Sinsabaugh and Moorhead, 1994; Yokoyama et al., 2017). The overall increase in C-acquisition and P-acquisition enzyme activities suggested that N addition may stimulate microbial demand for C and P. However, not all responses of enzyme activities to nutrient addition follow resource allocation theory. It is worth mentioning that N-acquisition (NAG) enzyme activity increased with N addition ($p < 0.05$; Fig. 2). NAG can be used to produce both N and C (Moorhead et al., 2012; Olander and Vitousek, 2000). In particular, when the dominant C sources are chitin, peptidoglycan, and protein compounds relative to cellulose, N addition can stimulate microbes to obtain C from these nutrients by increasing the activities of NAG (Allison et al., 2014).

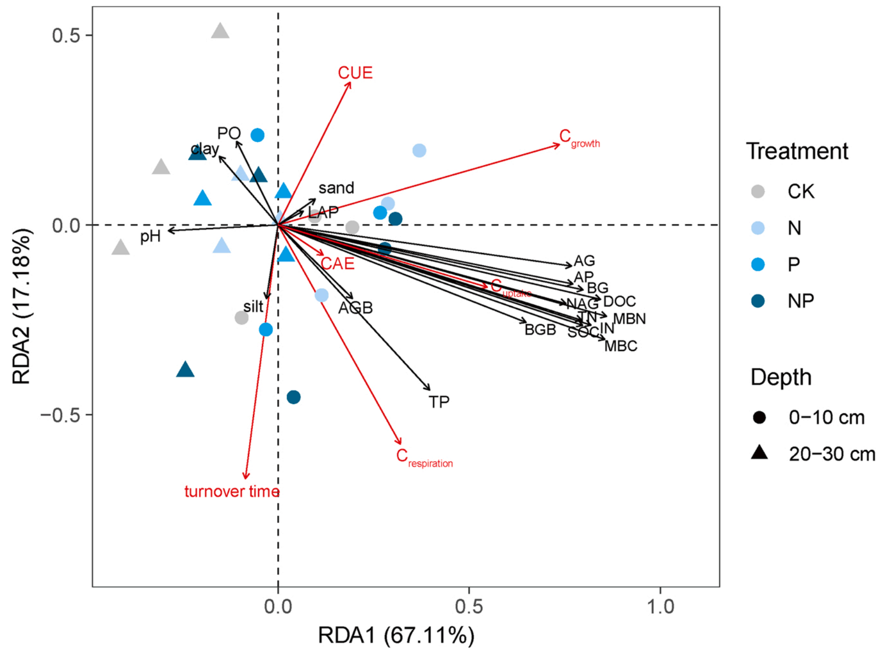

Fig. 6. Redundancy analyses (RDA) for the relationship between soil properties, plant biomass, and enzyme activities (black lines) and microbial physiological traits and efficiency (including CUE, CAE, and C_{growth}, $C_{respiration}$, C_{uptake}, and turnover time) (red lines) in the top- and subsoil with long-term nutrient addition. The different colors of points indicate four treatments, and the different shapes indicate two depths.

Table 1
Standardized regression coefficient of multiple stepwise regression for microbial CUE with soil properties and extracellular enzyme activities (n = 3). All variables were log_{10}-transformed.

Microbial parameters	R^2	p-value	Significant variables	Beta Coefficient
After short-term nutrient addition				
CUE	0.50	< 0.01	clay	-0.48
		< 0.01	AG	0.47
After long-term nutrient addition				
CUE	0.28	< 0.01	AG	0.56

Unlike hydrolases, PO activities did not change significantly after long-term nutrient addition ($p > 0.05$; Fig. 2). As long-term fertilization alleviates microbial energy and nutrient (C, N, and P) limitations (Chen et al., 2014; W.X. Liu et al., 2018), microbes do not need to degrade phenol compounds to obtain energy and nutrients, leading to unchanged PO activity.

4.2. Responses of microbial CUE to nutrient addition

Microbial CUE is a critical synthetic representation of microbial community metabolism (Manzoni et al., 2012). After short- and long-term nutrient addition, the measured microbial CUE was in the range of 20.70 ± 2.90 % and 54.58 ± 5.12 % (Fig. 3a, b), respectively. The results of long-term nutrient addition were similar to those of previous studies based on kinetic and metabolic considerations (Poeplau et al., 2019; Spohn et al., 2016; Widdig et al., 2020b), whereas those of short-term nutrient addition were lower than those in previous studies (Sinsabaugh et al., 2013; Spohn et al., 2016). The lower and undifferentiated microbial CUE implied that a 1-year nutrient addition was not sufficient to mitigate microbial nutrient limitation; microbes were still in an inefficient state of nutrient limitation (Chen et al., 2013; Wang et al., 2012). Unfortunately, the lower and undifferentiated microbial CUE may also be caused by the storage of the soils at − 80 °C for eight years, which may have affected the activities and functions of microbes.

We also found that microbial CUE did not significantly change with different treatments after both short- and long-term nutrient addition ($p > 0.05$; Fig. 3a, b). Widdig et al. (2020b) also found no change in microbial CUE in grassland soils with ≥ 7-year of N and P addition. They claimed that climate (temperature and precipitation) and soil texture played a dominant role in determining the microbial CUE (Widdig et al., 2020b; Zheng et al., 2019), with similar climate and soil texture (Tab. S3) may have resulted in the unchanged CUE in our study. In addition to environmental factors, a study indicated that the growth and respiration of microbes were both enhanced by N addition, which resulted in an unchanged CUE (Widdig et al., 2020b).

We also measured and compared microbial growth traits including C_{growth}, $C_{respiration}$, and C_{uptake}. The results showed that C_{growth}, the gross biomass production rate indicated by the C content of glucose-derived MBC, increased with short-term P and N + P addition to the topsoil, probably due to alleviated microbial P restriction (indicated by the increased P content in Figure S1). Consistent with a previous study (Luo et al., 2022), increased P availability stimulated microbial growth in a subalpine forest. Meanwhile, $C_{respiration}$, indicated by the respired C content derived from glucose, also exhibited an increasing trend, especially with N and P addition. These results reflect a generally positive response of microbes to short-term nutrient addition in the previous nutrient restriction state. Consequently, the simultaneous enhancement of C_{growth} and $C_{respiration}$ (although not significant) ultimately led to unchanged C_{uptake} and microbial CUE. In comparison, 10-year nutrient addition probably eliminated microbial nutrient restriction, and microbes did not follow the principle of preferential substrate for glucose in soils (Li et al., 2021). Thus, compared with CK, neither C_{growth} nor $C_{respiration}$ changed significantly, resulting in undifferentiated microbial CUE with long-term nutrient addition.

Furthermore, we found that AG played a dominant role in determining microbial CUE with both short- and long-term nutrient addition (Table 1). The positive correlation between AG and microbial CUE demonstrated the role of C-acquisition enzymes in decomposing C-enriched substrates to provide energy and maintain microbial growth (Jian et al., 2016; Liu et al., 2022). In addition, a negative correlation between clay and microbial CUE was also found in this study; SOC was prone to be protected by adsorption on clay minerals and formed stable minerals associated with organic matter with higher clay content (Six et al., 2004; Wang et al., 2022a), thereby lowering the availability of C substrates for microbial degradation (Islam et al., 2023; Pei et al., 2021), ultimately resulting in a lower CUE.

4.3. Responses of microbial CAE to nutrient addition

Microbial CAE was examined to evaluate the microbial efficiency of transforming new C into a relatively stable microbial necromass (Jia et al., 2017). In our study, microbial CAE did not change significantly in the top- and subsoil with short-term nutrient addition, whereas increased in the topsoil with long-term N addition (Fig. 4). Because microbial CAE was calculated with amino sugars derived from glucose relative to metabolized glucose, the unchanged $C_{respiration}$ and new amino sugars (indicated by ^{13}C-amino sugar; Tab. S4) resulted in similar microbial CAE with short-term nutrient addition.

The increased microbial CAE with long-term N addition in the topsoil indicated enhanced microbial necromass accumulation with higher N availability (indicated by increased IN with N addition, Tab. S2). As microbial necromass are N-enriched organic compounds (Heuck et al., 2015), they may be reutilized and/or degraded by microbes, especially under N limitation (Cui et al., 2020; Kaiser et al., 2014). Hence, the results highlight the positive effects of N availability on microbial CAE (Zhu et al., 2021), as reflected by the significant and divergent correlation between TP ($r = -0.54$, $p < 0.05$; short-term) and IN ($r = 0.40$, $p = 0.06$; long-term) with CAE (Fig. S2). The negative correlation between TP and CAE and the positive correlation between IN and CAE both implied the important role of N availability on microbial CAE, as a higher TP may increase microbial N limitation with 1-year nutrient addition (R.Z. Wang et al., 2022), which is also in line with the increased CAE with higher IN.

5. Conclusion

Based on a long-term N and P addition experiment in alpine grassland on the Tibetan Plateau, this study investigated the responses of microbial activities and physiological performances to short- and long-term nutrient addition. First, short-term N addition inhibited oxidase activities in the topsoil, which was in accordance with the microbial economic theory, whereas long-term nutrient addition exerted significant effects on hydrolase activities. Second, the short- and long-term nutrient addition did not significantly change the microbial CUE, whereas long-term N addition increased microbial CAE in the topsoil. We found that the C-acquisition enzymes (short- and long-term nutrient addition) and clay content (short-term nutrient addition) controlled microbial CUE, whereas TP (short-term nutrient addition) and IN (long-term nutrient addition) regulated microbial CAE. These findings provide evidence for elucidating the responses of microbes to increased N and P input from the perspective of microbial activities and functions in alpine grasslands.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

Data Availability

Data will be made available on request.

Acknowledgments

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Supplementary materials

The supplementary material related to this work can be found in the supplementary materials.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.still.2023.105657.

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