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Nutrient addition reduces carbon sequestration in a Tibetan grassland soil: Disentangling microbial and physical controls

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ABSTRACT

Nitrogen (N) and phosphorus (P) availability strongly affects carbon (C) cycling and storage in terrestrial ecosystems. Nutrient addition can increase C inputs into soil via increased above- and belowground plant productivity, but at the same time can accelerate organic matter decomposition in the soil. The mechanisms underlying these effects on soil organic C (SOC) dynamics remain unclear, especially in nutrient-limited alpine ecosystems that have been subjected to increasing N and P availability in recent decades. The aim of this study was to clarify the mechanisms underlying SOC decomposition and stabilization in an alpine grassland soil after four years of N and P additions. The soil aggregate size distribution, microbial community structure (lipid biomarkers), microbial C use efficiency (CUE) and microbial necromass composition (amino sugar biomarkers) were analyzed. Nutrient addition increased dominance of fast-growing bacteria (copiotrophs), while P addition alone intensified the competitive interactions between arbuscular mycorrhizal and saprotrophic fungi. These changes led to decreases in the microbial CUE of glucose by 1.6–3.5% and of vanillin by 8.5%, and therefore, reduced SOC content in the topsoil. The total microbial necromass remained unaffected by nutrient addition, but the contribution of fungal necromass to SOC increased. The increased abundance of arbuscular mycorrhizal fungi and fungal necromass under elevated N availability raised the mass proportion of soil macroaggregates (>250 μm) by 16.5–20.3%. Therefore, fungi were highly involved in macroaggregation following N addition, and so, moderated the SOC losses through enhanced physical protection. Overall, the complex interactions between microbial physiology (CUE), necromass composition (amino sugars) and physical protection (macroaggregation) in mediating SOC dynamics in response to nutrient enrichment were disentangled to better predict the capability of alpine grassland soils to act as a C sink or source under global change.

1. Introduction

The Tibetan Plateau is the highest and largest plateau on Earth, with a mean elevation of 4000 m above sea level and an area of approximately 2.5×10^6 km^2. Alpine grasslands cover more than 60% of the plateau surface and 7.4 Pg of carbon (C) are stored in the top 1 m of soil (Yang et al., 2008). The temperature rise in this region (0.3–0.4 °C per decade) is about twice as much as the global average rate (IPCC, 2013). This stimulates soil organic C (SOC) decomposition and thus increases the release of nutrients, especially mineral nitrogen (N) and phosphorus (P) (Mack et al., 2004). Meanwhile, the Tibetan Plateau has been experiencing increasing N deposition, ranging from 4.2 to 12.6 kg N ha^{-1} y^{-1} (Zhu et al., 2016).

The elevated N and P input can stimulate primary productivity by alleviating nutrient limitations for plant growth (Fay et al., 2015). This increased productivity then leads to increases in input by plant litter, roots and rhizodeposits, which in turn influence SOC dynamics (Fornara et al., 2013; Averill and Waring, 2018). However, the effects of nutrient

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addition on soil C sequestration are inconsistent and have been shown to be positive (Fornara et al., 2013; Ye et al., 2018), negative (Boot et al., 2016; Li et al., 2018) or neutral (Lu et al., 2011). These inconsistent effects are due to a number of mechanisms that affect SOC dynamics in different ways: (1) increase of C input by plant productivity (Fay et al., 2015); (2) acceleration of organic matter decomposition (Averill and Waring, 2018); (3) alteration of microbial C use efficiency (CUE) (Riggs and Hobbie, 2016); (4) accumulation of microbial necromass and shift in its composition (Griepentrog et al., 2014); and (5) formation of stable macroaggregates and so, better protection of organic matter from decomposition (Wilson et al., 2009). Therefore, a comprehensive analysis of these individual processes involved in SOC dynamics in Tibetan alpine grasslands can disentangle the interactions between N and P availability and C cycling under global change.

Microbial CUE is the ratio of C used for microbial growth and incorporation into tissues, to the total C taken up. It is a critical microbial physiological property that determines the fate of C in soils (Bradford et al., 2013; Geyer et al., 2016). The CUE reflects the partitioning of C between anabolic and catabolic processes within a microbial community (Sinsabaugh et al., 2013; Spohn et al., 2016). High CUE indicates efficient growth of microbes and an increased potential for C sequestration in soils. On the other hand, low CUE implies relatively greater loss of C via microbial respiration (Frey et al., 2013; Riggs and Hobbie, 2016; Sokol et al., 2019). Microbial CUE increases with the organic C quality of substrates (Bosatta and Ågren, 1999; Roller and Schmidt, 2015) and decreases with decreasing nutrient availability per available C (Devêvre and Horwáth, 2000). The latter phenomenon is correlated with the ecological stoichiometric theory. Microbes need to maintain the stoichiometry of their biomass by partitioning more assimilated C to respiration when they feed on substrates with an unfavorable stoichiometric ratio (Cleveland and Liptzin, 2007; Manzoni et al., 2012). Accordingly, nutrient addition could increase microbial CUE by decreasing the C-to-nutrient ratio of substrates (Manzoni et al., 2012; Li et al., 2015). It is generally presumed that fast-growing copiotrophs have a lower CUE, whereas slow-growing oligotrophs, with more conservative growth strategies, have a higher CUE (Keiblinger et al., 2010; Riggs and Hobbie, 2016). Although microbial community composition has been extensively investigated under nutrient addition (Leff et al., 2015), it remains unclear how nutrient enrichment affects microbial CUE and consequently, soil C sequestration.

Nutrient enrichment influences the abundance, activity and community structure of microbes (Fanin et al., 2015; Leff et al., 2015), and the subsequent accumulation of microbial-derived C (necromass) in soils (Kögel-Knabner, 2017). Microbial contribution to SOC is largely determined by microbial community dynamics through their effects on the balance between the formation and degradation of microbial byproducts (Six et al., 2006; Liang et al., 2017). Living microbial biomass only accounts for 1–2% of SOC, but microbes undergo the iterative processes of cell generation, growth and death; thus, microbial necromass can ultimately contribute up to 80% of SOC (Liang et al., 2011). Microbial necromass is more biochemically resistant to decay and more readily stabilized into the soil C pool than other SOC fractions (Kallenbach et al., 2016; Kopittke et al., 2018). Because of the small particle size of microbial necromass, they will be sequestered in micro- and nano-pores and are not available for access by living microorganisms and partly by their enzymes (Kuzyakov and Mason-Jones, 2018). Consequently, microbial necromass is considered to be an important constituent of stable SOC (Miltner et al., 2012; Liang et al., 2017).

Amino sugars are key components of microbial cell walls and they are stabilized in the soil after cell death. This makes it possible to use amino sugars as time-integrated biomarkers to assess the dynamics of microbial necromass and the contribution of necromass to SOC (Glaser et al., 2004; Amelung et al., 2008; Joergensen, 2018). Muramic acid (MurA) uniquely originates from bacterial peptidoglycan, while glucosamine (GlcN) is predominantly derived from the chitin of fungal cell walls but can also be found in bacteria and invertebrates (Parsons, 1981; Amelung, 2001; Appuhn and Joergensen, 2006). Although galactosamine (GalN) constitutes a significant portion (17–42%) of the total amino sugars found in the soil (Glaser et al., 2004; Joergensen, 2018), it is difficult to ascertain from which organisms it has originated (Engelking et al., 2007). Therefore, MurA and GlcN are used to indicate the respective contributions of bacteria and fungi to microbial-derived necromass (Joergensen and Wichern, 2008; Khan et al., 2016). Microbial necromass participates in the formation of soil aggregates and exerts more persistent effects on aggregate stabilization than living biomass (Chantigny et al., 1997; Six et al., 2006). In turn, the degree to which microbial necromass accumulates in soils largely depends on the extent of physical protection provided by soil aggregates (Six et al., 2006).

Recently, isotopic and spectroscopic studies have suggested that microbial accessibility to substrates, rather than the chemical recalcitrance of organic C, is the dominant factor regulating long-term C stability in soils (Schmidt et al., 2011; Dungait et al., 2012). Such “stable” SOC primarily results from physical occlusion in aggregates and chemical sorption in organo-mineral complexes (Cotrufo et al., 2013; Griepentrog et al., 2014; Ding et al., 2015). Nutrient addition can have a substantial impact on soil aggregate formation and stabilization (Tripathi et al., 2008; Wang et al., 2018). This, in turn, alters the soil porosity, water- and air-filled pore space, organic matter localization, oxygen diffusion and anaerobicity (Horn and Smucker, 2005; Zhang et al., 2015; Ebrahimi and Or, 2016). Changes in soil microbe microhabitats induced by aggregation can influence the structure and function of microbial communities, and thereby, SOC turnover (Gupta and Germida, 2015; Kravchenko et al., 2019). However, there is limited understanding of the interactions between microhabitats and microbial communities, and how these interactions control soil C storage in the context of increased nutrient availability.

Here, a four-year N and P addition experiment in an alpine grassland on the Tibetan Plateau was used to examine the effects of nutrient enrichment on soil C cycling and storage. The specific objectives of this study were to (1) investigate the impacts of nutrient addition on microbial CUE via changes in substrate stoichiometry and microbial community composition; (2) evaluate how nutrient addition affected the abundance of different groups within microbial communities, and thus the composition of microbial necromass, and its contribution to SOC; and (3) ascertain whether the expected changes in bacterial and fungal contributions caused by nutrient addition modified the soil aggregate composition and stability. It was hypothesized that nutrient addition would increase the microbial CUE by decreasing the C-to-nutrient ratio of substrates. Our previous work found that N and NP fertilization increased plant productivity and belowground C input (Luo et al., 2019). Therefore, it was hypothesized that (1) N addition would increase the living microbial biomass, hence stimulating the accumulation of microbial necromass and increasing its contribution to SOC, consequently promoting the formation of aggregates; and (2) P addition alone would have a weaker impact on these variables because grassland ecosystems are generally not limited by P (Vitousek et al., 2010).

2. Materials and methods

2.1. Site description

The field experiment was conducted at the Haibei Alpine Grassland Ecosystem Research Station in the northeastern Tibetan Plateau, Qinghai Province, China (37°37′N, 101°12′E; 3220 m above sea level). The region is characterized by a continental monsoon climate. The mean annual temperature is −1.2 °C, ranging from a mean monthly temperature of −14.8 °C in January to 9.8 °C in July. The mean annual precipitation is 489 mm. Approximately 80% of the precipitation occurs during the growing season from May to September. The soil is classified as a Cambisol (IUSS Working Group WRB, 2007), and the native plant community is dominated by *Kobresia humilis*, *Elymus nutans*, *Stipa aliena*, *Festuca ovina* and *Gentiana straminea*.

2.2. Experimental design and soil sampling

The N and P addition experiment was established in May 2011. Four treatments were included: no addition (Control), N addition (N, 100 kg N ha^{-1} y^{-1}), P addition (P, 50 kg P ha^{-1} y^{-1}), and combined N and P addition (NP, 100 kg N ha^{-1} y^{-1} plus 50 kg P ha^{-1} y^{-1}). Sixteen plots (6 m × 6 m) were arranged in a randomized block design including four replicate blocks. The widths of the buffer strips between blocks and between plots were 2 m and 1 m, respectively. N and P were applied in the form of urea and triple superphosphate, respectively. The fertilizers were divided into three equal parts and evenly distributed by hand onto the ground surface after sunset (for higher moisture levels) at the beginning of June, July and August each year during the growing season.

Five soil cores (5 cm diameter and 0–10 cm depth) were taken randomly from each plot at the end of July 2015 and then combined to form one composite sample. After all visible stones and plant residues were carefully removed with forceps, moist soil samples were gently broken apart along natural-break points and passed through an 8-mm sieve. After thorough mixing, the fresh soil samples were divided into three subsamples. One subsample was air-dried for the analysis of soil properties and amino sugars. The second subsample was used immediately for the analysis of dissolved organic C (DOC), mineral N, dissolved total N (DTN), aggregate fractionation and microbial CUE. The third subsample was stored at −20 °C for the analysis of microbial communities.

2.3. Soil properties analyses

Soil pH was measured in a 1:2.5 soil:water suspension using a glass electrode. The SOC and total N (TN) contents were determined using the wet oxidation-redox titration and the micro-Kjeldahl method, respectively (Lu, 2000). Mineral N (NH_4^+ and NO_3^-) and DTN were extracted from moist soil using 2 M KCl solution (with a soil:solution ratio of 1:5) and analyzed on a continuous-flow autoanalyzer (Skalar San^{++}, Breda, the Netherlands). The dissolved organic N (DON) was calculated as DTN − NH_4^+-N − NO_3^--N. The available P (AP) and total P (TP) in the soil were determined using $NaHCO_3$ extraction and H_2SO_4–$HClO_4$ digestion, respectively, and analyzed using the molybdenum blue method (Lu, 2000). DOC was extracted from moist soil using deionized water in a 1:5 soil:solution ratio, which was shaken for 30 min and centrifuged for 10 min at 5000×*g*. The supernatant was filtered through a 0.45-μm membrane filter and the filtrate was analyzed using a total organic carbon (TOC) analyzer (Multi N/C 3100, Analytik Jena, Germany).

Phospholipid fatty acids (PLFAs) were used to assess microbial community composition as described by Bossio and Scow (1998). In brief, lipids were extracted from 1 g of freeze-dried soil using a chloroform:methanol:citrate buffer (1:2:0.8, v:v:v). The phospholipids were separated from nonpolar lipids and converted into fatty acid methyl esters (FAMEs) prior to analysis. The FAMEs were quantified using a gas chromatograph (Agilent 7890, Santa Clara, USA) equipped with a flame ionization detector and a HP-Ultra 2 column (25.0 m × 200 μm × 0.33 μm). The FAMEs were identified using a MIDI Sherlock Microbial Identification System (MIDI Inc., Newark, USA). Peak areas were converted to nmol g^{-1} dry soil using the internal standard, methyl nonadecanoate (C19:0). The following PLFAs were used as representative markers of specific microbial groups: iso-, anteiso- and 10Me-branched PLFAs were used for Gram-positive bacteria (G^+ bacteria); monounsaturated and cyclopropyl PLFAs were used for Gram-negative bacteria (G^- bacteria); the PLFA, 16:1ω5c, was used for arbuscular mycorrhizal fungi (AMF); and the PLFAs, 18:1ω9c, 18:2ω6c and 18:3ω6c, were used for saprotrophic fungi (SF) (Bastida et al., 2013; Willers et al., 2015). The PLFAs, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0, were used for non-specific makers present in all microorganisms (Blagodatskaya et al., 2014). Bacterial PLFAs were calculated as the sum of G^+ and G^- bacterial PLFAs; fungal PLFAs were calculated as the sum of arbuscular mycorrhizal and saprotrophic fungal PLFAs; total microbial PLFAs were calculated as the sum of bacterial, fungal and non-specific PLFAs; and the ratios of fungi to bacteria (F/B), Gram-positive bacteria to Gram-negative bacteria (G^+/G^-) and arbuscular mycorrhizal fungi to saprotrophic fungi (AMF/SF) were calculated based on the PLFA biomass of each group.

2.4. Soil aggregate fractionation

Moist soil (<8 mm) was physically fractionated into four aggregate size classes using the wet-sieving method (Elliott, 1986). One hundred grams of moist soil (on an oven-dry basis) was soaked in deionized water on top of a 250-μm sieve for 5 min. The sieve was manually moved up and down by 3 cm, and the process was repeated 50 times during a period of 2 min. The fraction remaining on the 250-μm sieve was then collected and the weights were recorded. Water and the soil with a particle size <250 μm was then passed onto a 53-μm sieve, and the sieving procedure was repeated. Water and the remaining soil, which had passed through the 53-μm sieve, was centrifuged at 1800×*g* for 30 min at 4 °C and the supernatant was discarded. Accordingly, the macroaggregates (>250 μm), microaggregates (53–250 μm), and the silt plus clay fraction (<53 μm) were separated. The recovery of soil mass after fractionation was 98.5–99.7%. Organic C content contained in each soil aggregate was calculated by multiplying its mass proportion by the organic C concentration.

2.5. Measurement of microbial CUE

Microbial CUE was assessed by measuring the amount of a ^{13}C-labeled substrate (glucose or vanillin) incorporated into microbial biomass C (MBC) and respired as $^{13}CO_2$ (Riggs and Hobbie, 2016). Fresh soil samples (30 g, on an oven-dry basis) were treated with 60 μg C g^{-1} dry soil using 49.5 atom percent ^{13}C-labeled glucose or vanillin, representing labile and recalcitrant C substrates, respectively. The samples were then incubated for 24 h at 25 °C in airtight 500 mL glass jars. One set of samples received deionized water only and served as controls. Respired CO_2 was sampled at 0 and 24 h; 20 mL of the headspace gas of the jar was sampled with an airtight syringe to measure the CO_2 concentration and ^{13}C abundance using a gas chromatograph (Agilent 7890, Santa Clara, USA) coupled with an isotope ratio mass spectrometer (IRMS; MAT 253, Thermo Finnigan, Bremen, Germany). After incubation, soils were extracted to determine the amount of ^{13}C derived from the added substrate that had been incorporated into microbial biomass. The extraction was performed using the chloroform fumigation extraction method and the isotopic composition was determined using an elemental analyzer (Flash, 2000 Organic Elemental Analyzer, Thermo Fisher Scientific, Bremen, Germany) coupled with a continuous-flow IRMS (Delta V Advantage, Thermo Fisher Scientific, Bremen, Germany).

Microbial CUE was calculated as $MB^{13}C/(MB^{13}C + {}^{13}CO_2)$, where $MB^{13}C$ is the amount of substrate C incorporated into the microbial biomass and $^{13}CO_2$ is the substrate C respired as CO_2. The MBC was calculated as the difference in extractable C between the fumigated and correspondent non-fumigated soil, divided by a correction factor of 0.45 (Wu et al., 1990). The percentage of $^{13}CO_2$ and $MB^{13}C$ from the substrate was determined as: $\%C_{substrate} = (\delta_C - \delta_T)/(\delta_C - \delta_S) \times 100$, where δ_C is the $\delta^{13}C$ value of the respired CO_2 or MBC from the control; δ_T is the $\delta^{13}C$ of respired CO_2 or MBC in soils treated with substrate; and δ_S is the $\delta^{13}C$ of the labeled substrate. Substrate ^{13}C incorporated into the microbial biomass was determined using the following equation: $\delta^{13}C_{MBC} = (\delta^{13}C_{fum} \times C_{fum} - \delta^{13}C_{unfum} \times C_{unfum})/(C_{fum} - C_{unfum})$, where C_{fum} and C_{unfum} are the masses of C extracted from the fumigated and unfumigated samples, respectively; and $\delta^{13}C_{fum}$ and $\delta^{13}C_{unfum}$ refer to their corresponding $\delta^{13}C$ values.

2.6. Amino sugar analysis

Amino sugars, including GlcN, GalN and MurA, were analyzed according to the protocol of Zhang and Amelung (1996). Briefly, air-dried soil (<0.25 mm) containing approximately 0.3 mg N was hydrolyzed using 10 mL of 6 M HCl at 105 °C for 8 h. The hydrolysate was filtered through a Whatman 2 filter, dried on a rotary evaporator and re-dissolved in deionized water. The pH of the hydrolysate sample was adjusted to 6.6–6.8 using 1 M KOH and 0.01 M HCl. The pH-adjusted sample was centrifuged at 1500×*g* for 10 min. The supernatant was freeze-dried, the residues were washed with methanol and the recovered amino sugars were transformed into aldononitrile derivatives and extracted using 1.5 mL dichloromethane. Excess anhydride was removed with 1 M HCl and deionized water. The amino sugar derivatives were redissolved in 300 μL hexane and ethyl acetate solvent (v: v = 1:1) and separated using a Shimadzu Gas Chromatography Mass Spectrometer (GC-MS) QP 2010 PLUS (Shimadzu, Kyoto, Japan) equipped with an HP-5 fused silica column and a flame ionization detector. The amino sugars were quantified using the internal standard myo-inositol, which was added prior to hydrolyzation. The recovery efficiency of the amino sugars was monitored by adding *N*-methyl--D-glucamine as an internal recovery standard before derivatization. The total amino sugar concentration was calculated as the sum of GlcN, GalN and MurA.

Fungal- and bacterial-derived C, as an index for fungal and bacterial necromass, was calculated according to the methods of Appuhn and Joergensen (2006) and Engelking et al. (2007). Fungal C was determined by subtracting bacterial GlcN from total GlcN, assuming that MurA and GlcN occur at a 1–2 M ratio in bacterial cells: μg fungal C g^{-1} soil = (mmol GlcN g^{-1} soil – 2 × mmol MurA g^{-1} soil) × 179.2 g mol^{-1} × 9, where 179.2 is the molecular weight of GlcN and 9 is the conversion value of fungal GlcN to fungal C. Bacterial C (μg g^{-1}) was calculated by multiplying the content of MurA (μg g^{-1}) by 45. The total microbial necromass was estimated as the sum of fungal-derived C and bacterial-derived C.

2.7. Data analysis and statistics

Statistical analyses were carried out using the SPSS 19.0 software package for Windows (SPSS Inc., Chicago, IL, USA). The normality and homogeneity of the variance of the data were tested prior to analysis of variance (ANOVA) using the Kolmogorov-Smirnov test and the Levene statistic, respectively. Statistically significant differences among nutrient addition treatments were tested using a one-way ANOVA followed by the least significant difference test (LSD) at $p < 0.05$. Linear regression models were fitted to describe the relationships between microbial CUE and SOC content. Logarithmic regressions were used to determine the relationships between the mass proportion of macroaggregates and AMF abundance or fungal necromass.

3. Results

3.1. Soil abiotic and biotic properties

Addition of N alone or in combination with P increased soil NO_3^- and DON contents. Meanwhile, P additions (P and NP treatments) increased TP and AP ($p < 0.05$; Table 1). Compared with the Control, nutrient addition did not affect soil pH, DOC content and C/N ratio, but significantly decreased SOC and TN contents and C/P ratio. Nutrient addition increased the abundance of total microbial PLFAs, bacterial PLFAs, G^- bacterial PLFAs and fungal PLFAs ($p < 0.05$; Fig. 1), and reduced the G^+/G^- bacteria ratio. The G^+ bacterial PLFAs remained unaffected by nutrient addition. N additions (N and NP treatments) increased AMF abundance ($p < 0.05$). P addition alone increased the F/B ratio but decreased the AMF/SF ratio.

Table 1
Effects of N and P additions on soil properties.

Properties	Control	N	P	NP
pH	7.21 ± 0.10 a	7.11 ± 0.18 a	7.57 ± 0.17 a	7.13 ± 0.07 a
SOC (g C kg^{-1})	66.7 ± 1.0 a	59.9 ± 0.9 bc	57.8 ± 0.9 c	61.7 ± 0.8 b
TN (g N kg^{-1})	6.75 ± 0.10 a	6.08 ± 0.09 b	5.93 ± 0.10 b	6.10 ± 0.08 b
TP (g P kg^{-1})	0.79 ± 0.01 b	0.78 ± 0.01 b	0.99 ± 0.01 a	0.96 ± 0.03 a
C/N	9.9 ± 0.1 a	9.8 ± 0.1 a	9.8 ± 0.1 a	10.1 ± 0.1 a
C/P	84.2 ± 0.9 a	77.3 ± 0.9 b	58.4 ± 0.4 d	64.4 ± 2.6 c
DOC (mg C kg^{-1})	88.7 ± 1.1 a	96.7 ± 8.4 a	81.2 ± 2.0 a	93.0 ± 3.3 a
NH_4^+ (mg N kg^{-1})	3.67 ± 0.12 b	3.48 ± 0.09 b	4.19 ± 0.04 a	4.15 ± 0.21 a
NO_3^- (mg N kg^{-1})	22.3 ± 1.1 b	40.5 ± 5.1 a	17.1 ± 2.4 b	37.7 ± 5.7 a
DON (mg N kg^{-1})	33.8 ± 0.6 b	44.6 ± 1.5 a	36.4 ± 2.7 b	46.6 ± 3.5 a
AP (mg P kg^{-1})	7.0 ± 0.4 b	6.6 ± 0.5 b	45.3 ± 0.9 a	46.1 ± 1.2 a

Data are means ± standard errors ($n = 4$). Different letters within the same row indicate significant differences among treatments at $p < 0.05$. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; C/N, ratio of SOC to TN; C/P, ratio of SOC to TP; DOC, dissolved organic carbon; NH_4^+, ammonium; NO_3^-, nitrate; DON, dissolved organic nitrogen; AP, available phosphorus.

3.2. Distribution of soil aggregates and their organic C content

Macroaggregates accounted for 51.6–60.5% of the total soil mass in all the treatments. The macroaggregates were followed by microaggregates and the silt plus clay fraction (Fig. 2). Compared with the Control, the N and NP addition increased the mass proportion of macroaggregates by 16.5–20.3%, at the expense of a 15.1–18.9% decrease in microaggregates. The mass proportion of silt plus clay fraction remained constant. The organic C content in macroaggregates increased in the NP treatment and decreased in the P treatment (Fig. 2). Nutrient addition reduced the organic C content in microaggregates and silt plus clay by 13.1–20.8% and 7.3–16.0%, respectively.

3.3. Microbial CUE

The microbial CUE of ^{13}C-glucose was greater than that of ^{13}C-vanillin in all the treatments. Compared with the Control, nutrient addition decreased the CUE of ^{13}C-glucose by 1.6–3.5%, while P addition alone decreased the CUE of ^{13}C-vanillin by 8.5% (Fig. 3). The microbial CUE of ^{13}C-glucose and ^{13}C-vanillin increased with SOC content ($p < 0.01$; Fig. 4).

3.4. Amino sugars

The total amino sugars and GalN contents remained stable despite nutrient addition (Table 2). The GlcN content increased by 6.1–20.5%, whereas the MurA content decreased by 6.9–17.9% compared with the unfertilized Control. Compared with the Control, nutrient addition increased the fungal necromass by 8.2–23.5% but decreased the bacterial necromass by 6.9–17.9%, resulting in no change in the total microbial necromass (Fig. 5). The ratio of fungal necromass to bacterial necromass was 8.5–9.9 in the soil with added nutrients, which was higher than that in the Control soil (Fig. 5). Nutrient addition did not affect the contribution of bacterial necromass to SOC. However, the contributions of fungal necromass to SOC and total microbial necromass to SOC, increased by 24.7–33.4% and 17.7–23.9%, respectively.

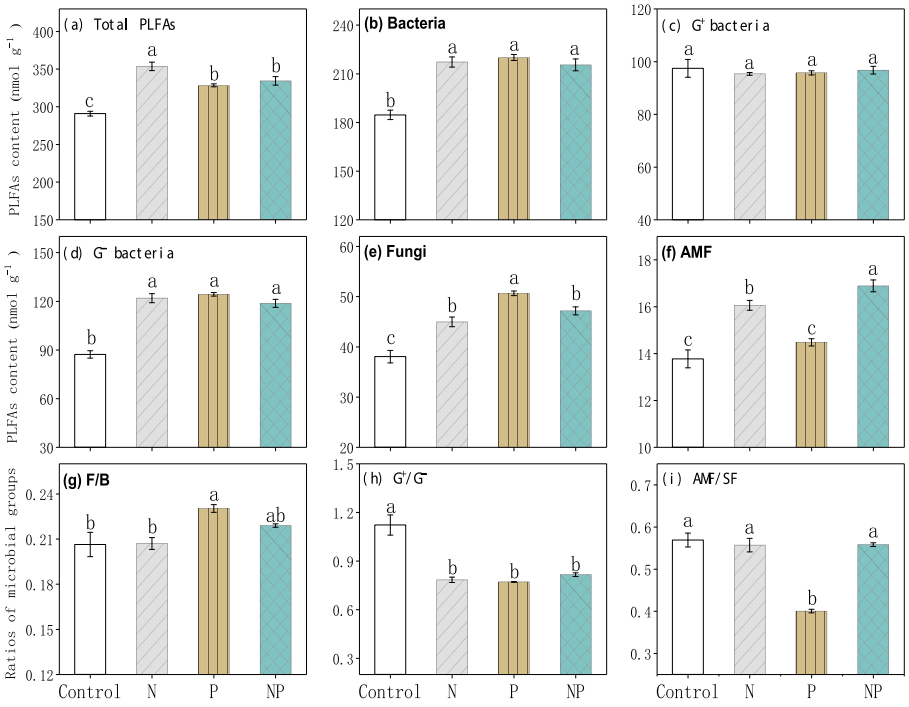

Fig. 1. Effects of N and P additions on abundance and ratios of soil microbial groups as indicated by phospholipid fatty acids (PLFAs). G^+ bacteria, Gram-positive bacteria; G^- bacteria, Gram-negative bacteria; AMF, arbuscular mycorrhizal fungi; F/B, ratio of fungi to bacteria; G^+/G^-, ratio of Gram-positive to Gram-negative bacteria; AMF/SF, ratio of arbuscular mycorrhizal fungi to saprotrophic fungi. Error bars represent the standard errors of the means ($n = 4$). Different letters indicate significant differences among treatments at $p < 0.05$.

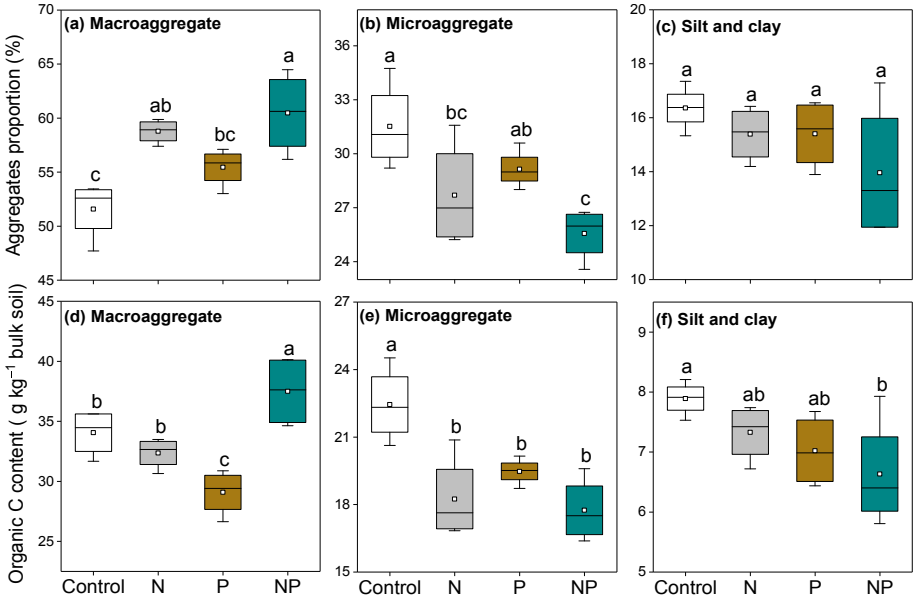

Fig. 2. Effects of N and P additions on distribution of mass proportions and organic C content of the (a, d) macroaggregates, (b, e) microaggregates and (c, f) silt plus clay fraction. Boundaries of boxes indicate the first and third quartiles, lines and squares within boxes represent the median and mean, respectively, and whiskers indicate the 10th and 90th percentiles ($n = 4$). Different letters indicate significant differences among treatments at $p < 0.05$.

4. Discussion

4.1. Effects of nutrient addition on microbial CUE

The measured microbial CUE was in accordance with the range of 0.5–0.8 that has been reported previously for various organic compounds (Frey et al., 2013; Jones et al., 2018). The microbial CUE of glucose was greater than that of vanillin. There are at least two explanations for this. Firstly, the breakdown of larger or more complex molecules requires the production of more enzymes and multiple oxidation steps. This could be associated with less efficient conversion of C into microbial biomass (Bosatta and Ågren, 1999). Secondly, the sampling time of 24 h was short for the complete uptake and utilization of more complex molecules. In general, high nutrient availability has been shown to increase microbial CUE, particularly when the substrate C/N ratio is low (Manzoni et al., 2012; Poeplau et al., 2019). This can be explained by the higher C demand required and energy losses that occur during N acquisition under N-limiting conditions (Schimel, 2003). In the present study, nutrient addition did not influence the C/N ratio in soil organic matter, but reduced the C/P ratio (Table 1). In contrast to our hypothesis, the continuous four-year nutrient addition decreased the CUE of glucose, while P addition alone also significantly decreased the

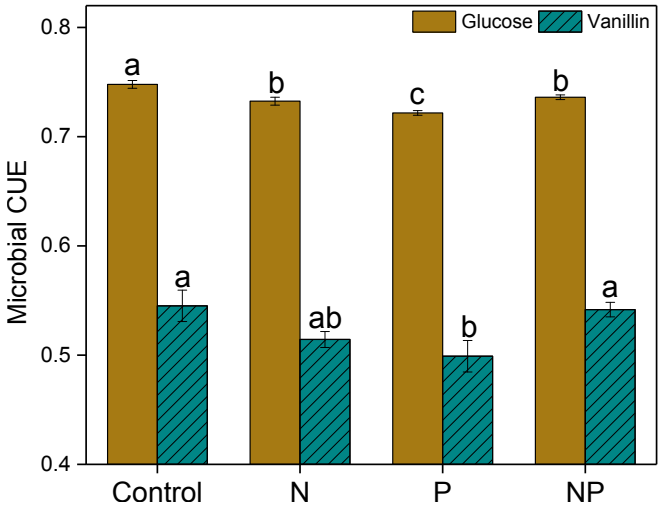

Fig. 3. Effects of N and P additions on the microbial carbon use efficiency (CUE) of ^{13}C-glucose and ^{13}C-vanillin. Error bars represent the standard errors of the means ($n = 4$). Different letters indicate significant differences among treatments at $p < 0.05$.

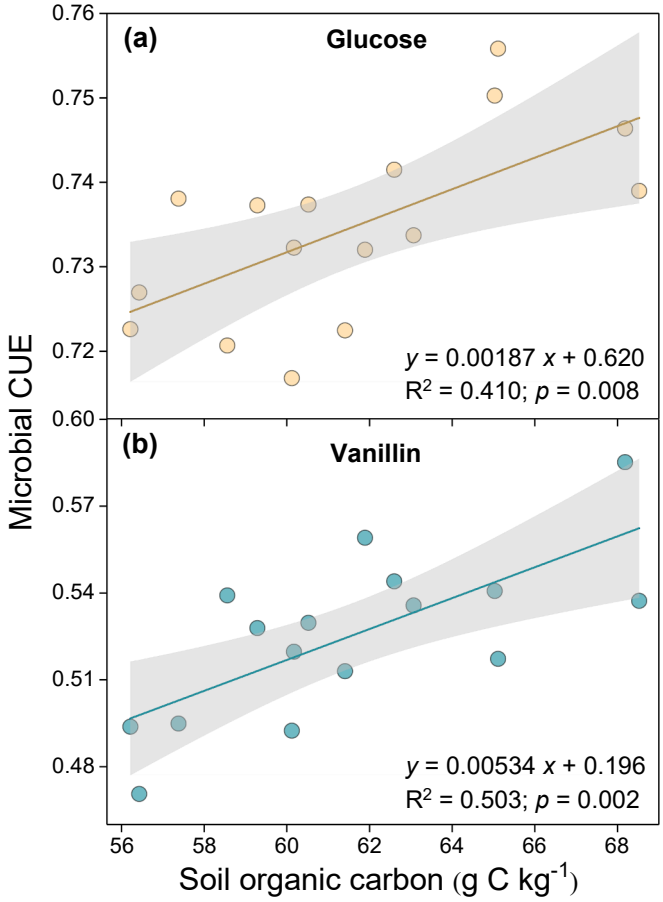

Fig. 4. Relationships between soil organic carbon content and the microbial carbon use efficiency (CUE) of (a) ^{13}C-glucose and (b) ^{13}C-vanillin. Shaded sections indicate the 95% confidence intervals of the regression lines.

CUE of vanillin compared with the unfertilized soil (Fig. 3).

Compared with the unfertilized Control, nutrient addition increased the bacterial abundance primarily by G$^-$ bacteria. This may be linked to several factors. First, the N and NP fertilization increased the aboveground plant biomass in the alpine grassland (Luo et al., 2019), which in turn delivered more photosynthates, such as exudates, root debris and plant litter into the soil (Li et al., 2015). Second, N addition reduced the C/N ratio of the photosynthates and so, increased the organic C quality of substrates for microbial utilization (Li et al., 2015). Third, P addition increased the available P content of the soil (Table 1). These changes were favorable for the growth of copiotrophs, such as G$^-$ bacteria, that tend to thrive on relatively labile organic C, and high P availability for maintaining a high density of P-rich ribosomes (Kramer and Gleixner, 2008; Fanin et al., 2015). These copiotrophs are generally characterized by rapid growth and low CUE (Kallenbach et al., 2015; Leff et al., 2015; Roller and Schmidt, 2015). This hence explains the decreased CUE of glucose observed for the whole microbial community under nutrient addition (Fig. 3).

Table 2
Effects of N and P additions on soil amino sugar contents (mg kg^{-1}) and ratios.

	Control	N	P	NP
Total amino sugars	5296 ± 194 a	5745 ± 276 a	5530 ± 257 a	6009 ± 369 a
GlcN	3363 ± 117 b	3742 ± 205 ab	3569 ± 161 ab	4054 ± 241 a
GalN	1746 ± 84 a	1830 ± 67 a	1809 ± 103 a	1796 ± 128 a
MurA	186 ± 7 a	174 ± 6 ab	153 ± 12 b	159 ± 9 ab
GlcN/MurA	18.1 ± 0.3 c	21.5 ± 0.9 b	23.6 ± 1.4 ab	25.4 ± 0.6 a

Data are means ± standard errors ($n = 4$). Different letters within the same row indicate significant differences among treatments at $p < 0.05$. GlcN, glucosamine; GalN, galactosamine; MurA, muramic acid.

The abundance of fungi that generally have a slow growth rate and high CUE (Keiblinger et al., 2010; Riggs and Hobbie, 2016), also strongly increased in the N and NP fertilized soils. This was due to increases in the abundances of arbuscular mycorrhizal and saprotrophic fungi. AMF can facilitate the uptake of soil nutrients, especially N and P, by their host plants through their hyphal network in exchange for photosynthetic C (Smith and Read, 2008). The result of this study differed from previous meta-analysis findings that the AMF abundance was reduced by 24% across all biomes globally under N addition (Treseder, 2004). Previous studies have shown that the grassland soils at the site used in the present study are seriously N limited (Song and Yu, 2015; Luo et al., 2019). Consequently, the addition of N alone alleviated the N limitation for plant growth, while aggravating soil P deficiency (Delavaux et al., 2017). Under these conditions, plants must invest more photosynthetic C into their associated AMF in exchange for additional P supply (Johnson, 2010). An alternative explanation for the increased abundance of AMF observed, is that *E. nutans* becomes an increasingly dominant plant species when soil N availability increases (Song and Yu, 2015), and more host plants are available to be colonized by AMF in N fertilized soil (Jiang et al., 2018). Unexpectedly, combined N and P addition also increased AMF abundance. Xie et al. (2014) demonstrated that P addition at a low rate led to increased AMF colonization, while at a high rate, impeded colonization in the soil. A two-year application of P at 50 kg P ha^{-1} y^{-1} along with 100 kg N ha^{-1} y^{-1} in a karst grassland has also been shown to stimulate the growth of AMF (Xiao et al., 2019). Thus, the P addition rate in the current study might have been insufficient to inhibit AMF growth.

The abundance of SF, rather than AMF, was increased under P addition alone. He et al. (2016) examined Tibetan alpine grassland soils sampled from the same site. They found that P addition alone increased the abundance of some non-mycorrhizal fungi (such as Sordariomycetes), whereas it decreased that of Glomeromycetes (a typical AMF group). The N/P stoichiometry in the soil is a key factor that influences the competitive advantage of fungal groups. Non-mycorrhizal fungi generally outperform AMF when the available N/P ratio is low (Chagnon and Bradley, 2013). In the present study, the ratio of mineral N to available P in the P treatment (0.5) was significantly lower than in the

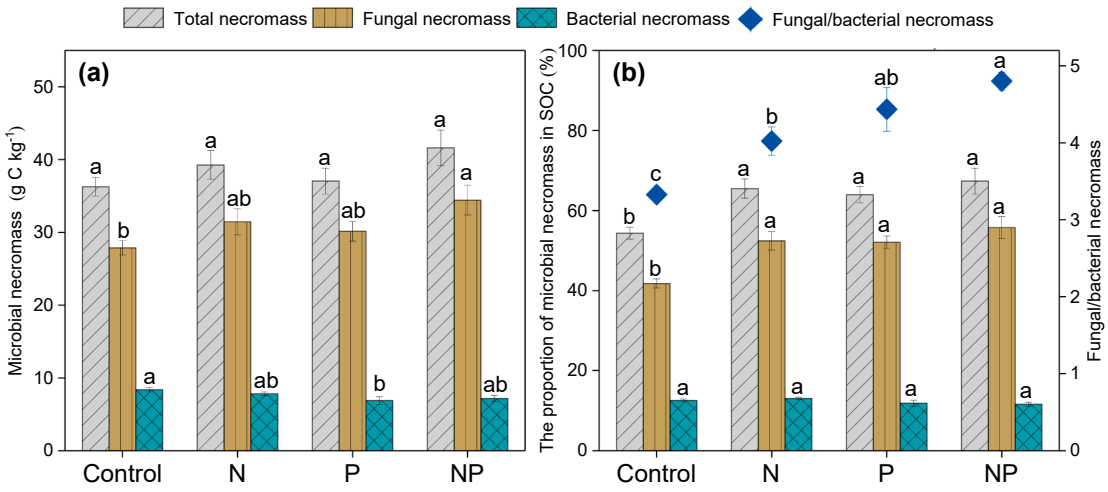

Fig. 5. Effects of N and P additions on (a) microbial necromass and (b) the ratios of microbial necromass to soil organic carbon (SOC) and fungal necromass to bacterial necromass. Error bars represent the standard errors of the means ($n = 4$). Different letters indicate significant differences among treatments at $p < 0.05$.

Control treatment (3.7; Table 1), which may have constrained the growth of AMF under P addition alone. Fungi are mainly involved in the depolymerization of recalcitrant C sources through the release of extracellular enzymes such as peroxidase and phenol oxidase (Schneider et al., 2012). It has been demonstrated that biotic interactions play an important role in microbial CUE (Frey et al., 2001; Buchkowski et al., 2017), and competitive interactions between fungal groups caused a decline in community-level CUE (Maynard et al., 2017). As such, it is likely that the increased competitive interactions between AMF and SF caused by N limitation resulted in more substrtate being respired through overflow respiration (Manzoni et al., 2012; Sinsabaugh et al., 2013), contributing to decline in the CUE of vanillin in the soil that was subjected to P addition alone.

The microbial CUE of glucose and vanillin increased with SOC content (Fig. 4). Microbes with a relatively higher CUE have greater potential for C sequestration by more efficiently converting substrates into microbial biomass (Sinsabaugh et al., 2013). This is especially true for fungi (Kallenbach et al., 2016). Poeplau et al. (2019) attributed a long-term positive effect of NPK fertilization on SOC sequestration in temperate grasslands to increased CUE. Nutrient addition decreased the microbial CUE of glucose and vanillin (Fig. 3). Thus, it can be argued that the dynamics of C sequestration in the studied grassland soil under nutrient addition might depend on the microbial CUE as a result of shifts in the microbial community structure: C sequestration may increase with increasing microbial CUE and decrease with decreasing microbial CUE. In this study, the nutrient-induced decrease in microbial CUE caused a greater proportion of labile and recalcitrant C to be mineralized and lost via microbial respiration, thereby lowering soil C sequestration in the alpine grassland (Kallenbach et al., 2015; Riggs and Hobbie, 2016).

4.2. *Effects of nutrient addition on microbial necromass and aggregate formation*

The total amount of microbial necromass remained unaffected by nutrient addition. However, nutrient addition increased the ratio of total microbial necromass to SOC from 54% (Control) to 64–67%, due to the decrease in SOC (Fig. 5). This ratio was higher than in tropical forest soils (25–45%; Zhang et al., 2016), but was similar to arable and grassland soils (50%; Khan et al., 2016) and wetland soils (50–70%; Ding et al., 2019). The ratio of fungal necromass to SOC increased after nutrient addition (Fig. 5b). This indicates that microbial-, and especially fungal-, derived C inputs become prominent at the expense of plant-derived C in terms of C storage in alpine grassland soils under nutrient enrichment, resulting in a change in the SOC composition.

Although the different N and P additions increased the bacterial abundance, the bacterial necromass content did not increase; it even significantly decreased with P addition alone (Fig. 5a). Similarly, Chen et al. (2018) observed that N addition had little impact on bacterial necromass in paddy soils during a 100-day incubation of rice residue. Bacterial necromass can serve as a "capacitor" in SOC turnover, playing an active role in adjusting the microbial stoichiometric demand (He et al., 2011). P addition alone can aggravate C limitation for microbes (Spohn and Kuzyakov, 2013). Therefore, it is possible that in this study, bacterial necromass was degraded gradually to compensate for the increased microbial C demand, leading to decreases in bacterial necromass in the P treatment.

In contrast, N addition alone slightly and NP addition significantly accelerated the accumulation of fungal necromass, and consequently increased the ratios of fungal to bacterial necromass (Fig. 5). Similarly, four years of N deposition (70 kg N ha^{-1} y^{-1}) has been shown to elevate fungal necromass in forest soils (Griepentrog et al., 2014). Van Groenigen et al. (2007) found that the amount of fungal necromass in a grassland ecosystem was primarily correlated with fungal abundance. N addition stimulated the proliferation of fungi (Fig. 1e), which in turn increased the fungal necromass. Fungal fragments are larger than bacterial, and have lower surface area to volume ratios associated with thicker cell walls that efficiently form macromolecular aggregations (Schweigert et al., 2015). This is indicative of the high stability of fungal necromass (Amelung et al., 2008). These fragments have been shown to preferentially protect against microbial decomposition through their interactions with clay and occlusion within aggregates, which contributes to the physiochemical protection of fungal necromass (Six et al., 2006; Fernandez et al., 2016). However, the smallest increase in fungal necromass compared with the unfertilized Control, was observed in the soil that was treated with P fertilization alone. Khan et al. (2016) pointed out that the higher the contribution of AMF and the lower the contribution of SF to the fungal community, the more fungal necromass can be sequestered. Consequently, it can be argued that the low accumulation of fungal necromass after P addition alone may partially result from the lower ratio of AMF/SF compared with the Control.

Consistent with our initial hypothesis, N addition fostered the formation of macroaggregates, while P addition alone did not (Fig. 2). Tripathi et al. (2008) and Wang et al. (2018) observed a similar stimulation effect of N addition on soil aggregation. A regression analysis revealed that the mass proportion of macroaggregates was closely correlated with the AMF abundance and fungal necromass (Fig. 6), as found by Wilson et al. (2009) and Murugan et al. (2019). The increased

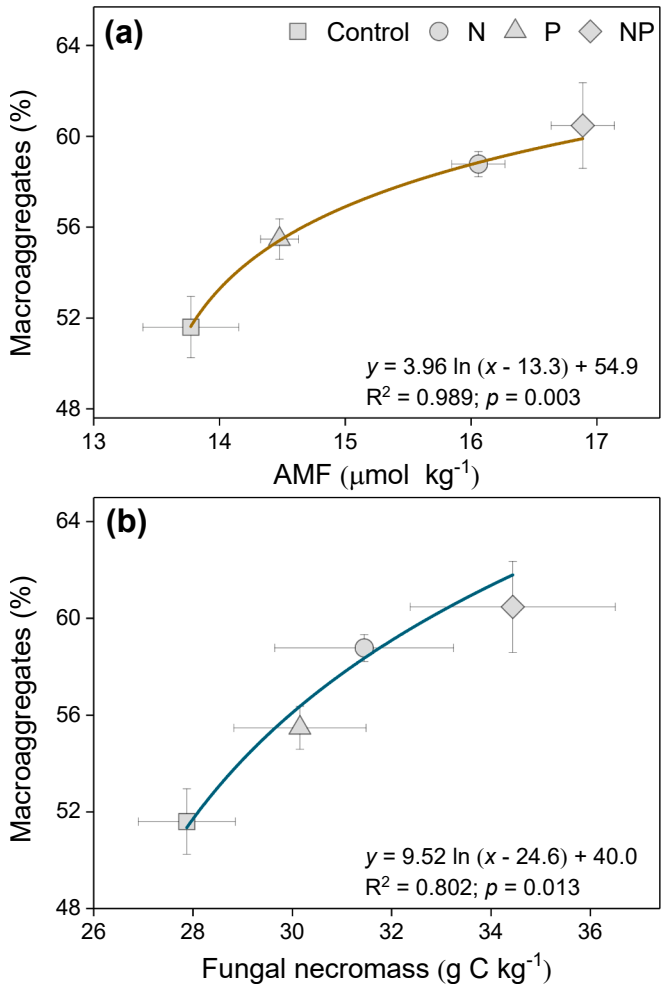

Fig. 6. Relationships between the mass proportion of macroaggregates and (a) the abundance of arbuscular mycorrhizal fungi (AMF) and (b) fungal necromass.

fungal hyphae and the associated cell wall residues under N addition acted as binding agents by enmeshing, cross-linking and adhering primary mineral particles, organic matter and microaggregates, thereby reinforcing formation and stabilization of the macroaggregates (Ding et al., 2015; Rillig et al., 2015; Lehmann et al., 2017; Morris et al., 2019). The macroaggregation could impede microbial and enzymatic access to the substrates within aggregates (Dungait et al., 2012), and cause the development of anaerobic microsites and suppress the proliferation of aerobes, consequently limiting the aerobic decomposition of organic matter to CO_2 (Blagodatsky and Smith, 2012; Zhang et al., 2015; Keiluweit et al., 2017). Compared with the Control, N addition caused less depletion of SOC content relative to P addition alone (Table 1). Therefore, the formation of macroaggregates driven by fungi may reduce SOC loss via enhanced physical protection of SOC from microbial decomposition under N addition (Tripathi et al., 2008; Wilson et al., 2009).

4.3. Implications for alpine grassland soil C sequestration

The increased SOC loss in response to nutrient addition (Table 1) is not common for a grassland ecosystem, given that N and NP addition increased plant productivity and belowground C input, while P addition alone had no effect (Luo et al., 2019). This is different from the nutrient-induced increases in soil C sequestration that have previously been found in temperate grasslands (Ye et al., 2018; Poeplau et al., 2019). Some studies have also reported that nutrient addition lowered SOC in an alpine meadow (Li et al., 2018), the Arctic tundra (Mack et al., 2004) and subalpine forests (Boot et al., 2016). The Nutrient Network global change experiment was performed across a range of climate and soil types in grasslands worldwide. Based on this experiment, Crowther et al. (2019) concluded that nutrient enrichment has the potential to drive soil C loss in many high-latitude Arctic and sub-Arctic regions. Given that a large SOC exists in alpine and Arctic regions, these responses highlight the vulnerability of soil C storage to anthropogenic nutrient deposition. These anthropogenic additions can lead to potential changes in the terrestrial C balance and positive feedback to climate warming. Our field study was carried out over a relatively short timescale (four years), and only reflected initial, short-term SOC responses to nutrient enrichment. In a sugar maple-basswood ecosystem, Waldrop et al. (2004) observed that N deposition (80 kg N ha^{-1} y^{-1}) reduced SOC content by 20% in the first three years, but it increased the SOC content to the same level as that in the control soil after six years (Grandy et al., 2008). Thus, the SOC decrease after four years of N addition to alpine grasslands may be temporary, and further study is required to monitor the long-term SOC dynamics under nutrient enrichment.

5. Conclusions

In this study, the main effects of different factors on SOC decomposition and stabilization were investigated in alpine grassland soils following N and P additions (Fig. 7). The four years of nutrient addition decreased the microbial CUE of glucose, while P addition alone decreased the CUE of vanillin. This was due to shifts in the bacterial communities to more G^- bacteria and an increase in competitive interactions between AMF and SF. The lower microbial CUE of glucose and vanillin was related to the lower SOC content under nutrient enrichment. This indicated that short-term nutrient addition caused greater losses of labile and recalcitrant organic C by microbial respiration, thereby lowering soil C sequestration. Nutrient addition increased the contribution of fungi to SOC, which was indicated by increases in the ratios of fungal to bacterial necromass and fungal necromass to SOC. The addition of N increased the AMF abundance and fungal necromass, which accelerated the formation of macroaggregates, consequently decreasing SOC decomposition by enhancing physical protection. The decreased SOC content observed after N addition in alpine grasslands may be temporary, and long-term field studies are vital to evaluate whether the soil serves as a C sink or source when N and P availabilities increase.

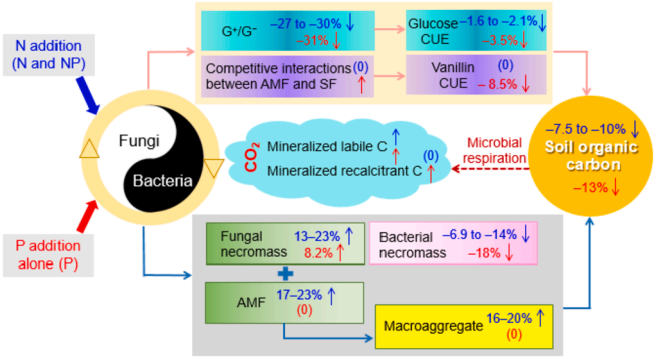

Fig. 7. A conceptual diagram illustrating the main effects of various factors on soil organic carbon decomposition and stabilization in response to N and P additions in alpine grassland. AMF, arbuscular mycorrhizal fungi; SF, saprotrophic fungi; G^+/G^-, ratio of Gram-positive to Gram-negative bacteria; CUE, microbial carbon use efficiency. The short arrows↓and↑, and (0) represent decrease, increase and no change in soil C processes in response to N and P additions, respectively. The blue and red symbols and numbers denote the processes influenced by N addition and P addition alone, respectively. The numbers denote the increase or decrease in % compared to the unfertilized Control soil. No percentage before the arrows indicates that the processes could not be quantified in this study.

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 paper.

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