



Long-term litter type treatments alter soil carbon composition but not microbial carbon utilization in a mixed pine-oak forest

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Abstract Changes in litter and nutrient inputs into soil could have significant consequences on forest carbon (C) dynamics via controls on the structure and microbial utilization of soil organic C (SOC). In this study, we assessed changes in physical fractions (250–2000 μm , 53–250 μm , and $< 53 \mu\text{m}$ soil aggregates) and chemical fractions (labile, intermediate and recalcitrant pools) of SOC in the top 20 cm mineral soil layer and their influences on microbial substrate utilization after eight years of experiment in a mixed pine-oak forest. The litter treatments included: control (L_{con}), litter removal (L_{nil}), fine woody litter addition (L_{woody}), leaf litter addition (L_{leaf}) and a mix of leaf and fine woody litter (L_{mix}). Nitrogen (N) addition (application rates of 0, 5 and 10 $\text{g N m}^{-2} \text{ year}^{-1}$, respectively) was also applied. We found that complete removal of forest-floor litter (L_{nil}) significantly reduced the pool sizes of all SOC fractions in both the

physical and chemical fractions compared with treatments that retained either leaf litter (L_{leaf}) or mixture of leaves and fine woody materials (L_{mix}). The type of litter was more important in affecting SOC fractions than the quantity of inputs; neither the level of N addition rate nor its interaction with litter treatment had significant effects on both physical and chemical SOC fractions. Microbial respiration differed significantly among the treatments of varying litter types. However, the effectiveness of microbial C utilization inferred by microbial C use efficiency and biomass-specific respiration was not affected by either the litter treatments or N addition. These results suggest that despite significant changes in SOC composition due to long-term treatments of forest-floor litter and N addition in this mixed pine-oak forest of temperate climate, microbial C utilization strategies remain unaffected.

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Introduction

The amount of carbon (C) stored in soil worldwide far exceeds the amount of carbon stored in plants and the atmosphere combined (Scharlemann et al. 2014), and

it is very sensitive to global change factors, such as CO₂ fertilization effect-enhanced plant growth and nitrogen (N) deposition-induced changes in soil nutrient availability (Thornton et al. 2007). Plant litter play a vital role in determining soil organic C (SOC) storage and cycling in forest ecosystems, as some of them are translocated to mineral soil as particulate or dissolved organic matter, and transformed to various physical components (i.e., organic C occluded in soil aggregates of different sizes) and chemical fractions (i.e., labile and recalcitrant organic C determined by chemical structure) (Schmidt et al. 2011; Cotrufo et al. 2013). However, the effects of litter on SOC dynamics are inconsistent, depending on litter quantity and quality and soil conditions (von Haden et al. 2019). For example, the associated processes of mineralization and stabilization of lignin, cellulose and other compounds through a series of actions by microorganisms are the major pathways of litter decomposition; the performance of these processes is dependent of the abiotic conditions like soil temperature and moisture, and biotic factors such as chemical composition of litter and soil organisms (Krishna and Mohan 2017). A modelling study by Castellano et al. (2015) suggested that the effect of litter quality on SOC stabilization depends on the extent of soil C saturation. von Haden et al. (2019) found that high litter input and low litter C:N ratios (which is typically associated with higher litter quality) stimulated SOC storage in soil aggregates and mineral-associated fractions. Fresh litter inputs may also cause soil priming (Kuzakov et al. 2000), such that soil C replenishment by new litter input may be offset by the primed decomposition of native soil C (Sayer et al. 2011; Cotrufo et al. 2013; Lajtha et al. 2018). But it remains an open question if the litter inputs of differential quality and quantity would affect the stability of soil carbon storage through modification of the SOC composition. Elucidation of the associations of litter inputs with various physical and chemical SOC fractions will help gain better insight in the regulation of SOC dynamics by litter production and variations.

Nutrient availability represented by N is another important factor modulating the distribution and turnover of SOC fractions (e.g., physical aggregation and chemical recalcitrance). Soil N availability directly affects soil pH, enzyme activities, and microbial substrate availability and community structure (Treseder 2008). For example, soil acidification

caused by N deposition could inhibit soil microbial metabolism (Treseder 2008). In N-limited systems, however, N deposition may relax N limitation (Reay et al. 2008), thus accelerating microbial utilization of labile organic C (Tian et al. 2016). In addition, increased soil N availability may also directly increase litter decomposition due to the increased soil enzyme activity (Manning et al. 2008). However, it is still uncertain whether and how N availability interacts with litter quantity and quality to affect soil physical and chemical C fractions in situ under natural environmental conditions. A major reason for such uncertainty is due to the fact that the total impacts on SOC dynamics result from the sum of effects across physically and chemically distinct SOC pools (Neff et al. 2002), which may respond differently to changing conditions. Different SOC pools have distinct turnover timescales due to physical protection which is partly determined by soil structure and mineralogy (Schmidt et al. 2011), biochemical resistance to degradation, and/or selective utilization by different microbial functional groups (von Lützow et al. 2006; Shen et al. 2018; Luo et al. 2020). Overall, our understanding on how SOC composition responds to changes in long-term C and nutrient inputs needs improvement and to some extent, constrains our ability to reliably predict long-term SOC dynamics.

Potential SOC composition changes under altered C and nutrient inputs may shape the structure and functioning of microbial decomposer communities (Cotrufo et al. 2013). Several studies have shown that soil microorganisms can adapt to changes in C substrates by adjusting their community structure, metabolism, and substrate utilization strategies (Goldfarb et al. 2011; Sinsabaugh et al. 2013). Apart from the regulating effects of SOC composition on the microbial processes, microbial metabolism changes in turn may also affect SOC composition via changed microbial necromass, which is an important component of SOC (Kallenbach et al. 2015, 2016; Ludwig et al. 2015; Liang et al. 2017). Due to the complexity of SOC compound fractions and microbial decomposition processes involved in the decomposition of those SOC fractions, the connections between the dynamics of SOC fractions and microbial metabolisms are rarely investigated.

We conducted a long-term experiment with altered litter inputs and varying level of N addition in a mixed pine (*Pinus tabulaeformis* Carr.) and oak (*Quercus*

wutaishanica Mayr) forest of northern China. The litter treatments included: control (L_{con}), litter removal (L_{nil}), fine woody litter addition (L_{woody}), leaf litter addition (L_{leaf}) and a mix of leaf and fine woody litter (L_{mix}). N addition was applied at 0, 5 and 10 g N m⁻² year⁻¹ in the form of urea. The objectives of our study were to address the question that how changes in litter and N inputs would influence physical and chemical component fractions of SOC, and to determine whether and how potential changes in SOC composition would affect microbial C utilization. We hypothesized that increases in both quantity and quality (e.g. woody materials vs leaves) of forest-floor litter inputs alter SOC composition, and this alteration is further modulated by N addition because of increased nutrient availability for microbial energy acquisition (i.e., assimilation of different SOC fractions). Due to microbial C use efficiency (CUE) increases with increasing soil C and N availability (Sinsabaugh et al. 2013), we further hypothesized that microbial CUE is enhanced under the conditions of high substrate (C and N) availability after eight years of experimental treatments.

Materials and methods

Study site and experimental design

This study was conducted at a natural forest site in Taiyue Mountains of Shanxi province, China (latitude 33°39'N, longitude 112°07'E, elevation 1500 m a.s.l.). It is in a region with a warm temperate and semi-humid continental monsoon climate, with a mean annual temperature of 8.6 °C, and a mean annual precipitation of 662 mm. About 60% of the precipitation falls during the period from July to September (Sun et al. 2016). The soil belongs to brown forest soils developed from limestone with a pH of 6.85, 7.02 and 7.47 in 0–5 cm, 5–10 cm and 10–20 cm soil layers, respectively. The soil type is classified as Alfisols according to the international soil classification system (IUSS Working Group WRB 2014). The surface soil (0–20 cm) contains 68.73 g kg⁻¹ organic C and 4.84 g kg⁻¹ total N. The litter is typically composed of leaves, fine branches, and fruiting bodies (mainly pinecones and oak seeds) on forest floor, with average density of 366, 158 and 253 g m⁻² year⁻¹, respectively.

A field experiment with a randomized block design involving four types of litter treatments and three levels of N addition rate was established in 2009 in a mixed pine (*P. tabulaeformis*) and oak (*Q. wutaishanica*) stand with very little understory growth. There were five blocks laid out over a distance of 40 m along the contour and 10 m along the slope, with adjacent blocks separated by a 1 m gap. Within each block, combinations of litter type treatments and N addition rates were randomly assigned to 12 2 × 2 m plots separated by minimum distance of 0.5 m. The four litter type treatments were aligned with the modified DIRT (the detrital input and removal treatment) project (Lajtha et al. 2018), including (1) complete removal of forest-floor litter (L_{nil}), (2) placement of fine woody litter of twice the natural density (L_{woody}), (3) placement of leaf litter of twice the natural density (L_{leaf}), and (4) placement of mixed leaf and fine woody litter of twice the natural density (L_{mix}). Specifically, all forest-floor litter on L_{nil} plots were collected and transferred to L_{mix} plots, whilst leaf litter and fine woody litter were sorted and exchanged between L_{woody} and L_{leaf} plots. Another group of five 2 × 2 m plots adjacent to the treatment plots were set up as controls of natural site conditions, designated as L_{con} . As a result of the differential litter regimes, the litter type treatment plots are distinguishable in terms of litter quality and quantity. For example, litter dry mass (kg m⁻²) in L_{woody} plots was significantly lower than that in L_{leaf} and L_{mix} plots. However, the litter in L_{woody} plots had significantly higher C:N and acid-insoluble materials (AIM) to nitrogen (AIM:N) ratios than in L_{mix} and L_{leaf} plots; whilst the C:N and AIM:N ratios were comparable between L_{leaf} and L_{mix} plots (Sun et al. 2016). Three levels of N addition rate were interactively incorporated with the litter type treatments, including 0 (N_0), 5 (N_5) and 10 (N_{10}) g N m⁻² year⁻¹, applied as urea. At each time of plot maintenance, one quarter of total urea required to be applied each year was mixed with ~ 500 g soil collected from the corresponding plot, and sprinkled evenly across the plot. The plots were tended, mainly for sorting the litter and applying N, four times a year, in April, June, August, and October, respectively.

Soil sampling and physicochemical properties

In August 2017, the litter layer was carefully cleared before soil sampling. Soil samples were extracted

using a soil auger with 3 cm inner diameter from 5 random points on each plot, and mixed to yield one composite sample per plot. Each core was split into three soil layers: 0–5 cm; 5–10 cm and 10–20 cm. Visible litter, roots, and stones were carefully removed. The soil samples were stored in airtight polypropylene bags and placed in a cooler filled with ice-cubes during transportation to the laboratory. The composite sample was divided into two subsamples in laboratory: one air-dried for determining soil physicochemical properties, and the other temporarily stored at $-20\text{ }^{\circ}\text{C}$ for later measurements of microbial properties.

Using the air-dried samples, soil total N (TN) was analyzed using semimicro-Kjeldahl (UDK159, VELP, Italy). Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were analyzed colorimetrically by a continuous flow analyzer (SEAL AA3, Norderstedt, Germany). Dissolved organic C and N (DOC and DON) was determined by measuring C and N concentration in the $0.5\text{ mol L}^{-1}\text{ K}_2\text{SO}_4$ extract solution (soil:solution = 1:4) after filtering through a membrane filter with $0.45\text{-}\mu\text{m}$ pores using Multi 3100 N/C TOC analyzer (Analytik Jena, Germany).

Physical and chemical fractionations of SOC

Using the air-dried samples, soil aggregates were separated based on the method by Six et al. (1998). Briefly, 20 g soil was placed in a nest of sieves of $2000\text{ }\mu\text{m}$, $250\text{ }\mu\text{m}$ and $53\text{ }\mu\text{m}$. Then the macro ($250\text{--}2000\text{ }\mu\text{m}$), micro ($53\text{--}250\text{ }\mu\text{m}$) and mineral size ($< 53\text{ }\mu\text{m}$) aggregates were separated and collected by shaking the sieves with 12 glass beads of 6 mm. Organic C content in these aggregates (named Macro-C, Micro-C and Mineral-C, respectively) was measured using the Vario ELIII Elemental Analyzer (Elementar, Germany). The percentage of Macro-C (fMacro-C), Micro-C (fMicro-C) and Mineral-C (fMineral-C) in total SOC (TOC) was calculated. The soil samples were treated with diluted HCl to remove the inorganic C prior to the determination.

Two-step hydrolysis with H_2SO_4 (Rovira and Vallejo 2002) was applied to determine the chemical fractions of SOC. Briefly, 500 mg of ground soil sample was hydrolyzed with 20 mL of $2.5\text{ mol L}^{-1}\text{ H}_2\text{SO}_4$ at $105\text{ }^{\circ}\text{C}$ for 30 min. Then the hydrolyzed solution was centrifuged for 10 min at a speed of 5000 rpm min^{-1} . The supernatant hydrolysate was

collected to a 50 mL tube. The soil residues were further rinsed with 20 mL of deionized water and centrifuged, and the supernatant hydrolysate added to the 50 mL tube. Carbon content in the hydrolysate of the 50 mL tube was measured using Multi 3100 N/C TOC analyzer. This carbon was treated as labile organic C (LOC) as it mainly includes polysaccharides of plant- and microbe-origins, which usually decompose rapidly and readily available for microbial consumption (Oades et al. 1970). The remaining soil residues were hydrolyzed and shacked overnight with 2 mL of $13\text{ mol L}^{-1}\text{ H}_2\text{SO}_4$ at room temperature, and then deionized water was added to dilute H_2SO_4 concentration to 1 mol L^{-1} . The diluted hydrolysate was kept at $105\text{ }^{\circ}\text{C}$ for 3 h, and then centrifuged for 10 min. The supernatant hydrolysate was measured for C content, and regarded as intermediate organic C (IOC). The remaining soil residues were measured for C content using Vario EL III Elemental Analyzer, which is the C retained in the soil that cannot be hydrolyzed by the two preceding steps and treated as recalcitrant organic C (ROC) (Oades et al. 1970), i.e., mineral-associated or chemically recalcitrant organic C. We also calculated the proportion of LOC, IOC and ROC in TOC (i.e., fLOC, fIOC, and fROC, respectively).

Measurements of soil microbial biomass and respiration

Using the freeze-stored soil samples, microbial biomass C (MBC) was measured by the fumigation-extraction method. Briefly, 20 g of equivalent dry soil was exposed to chloroform vapor for 24 h and then extracted with 80 mL of $0.5\text{ mol L}^{-1}\text{ K}_2\text{SO}_4$, shaken for 30 min, and then filtered using Whatman filter paper no.42. Organic C concentration in the extracts was measured by a Multi 3100 N/C TOC/TON analyzer (Analytik Jena, Germany). MBC was calculated as the difference in extractable C before and after fumigation using a conversion factor of 0.45 (Vance et al. 1987). Microbial respiration (MR) was measured by determining CO_2 evolution rates by incubating 25 g fresh soil at 60% water holding capacity at $25\text{ }^{\circ}\text{C}$. CO_2 was collected into 10 mL of $0.1\text{ mol L}^{-1}\text{ NaOH}$ solution and titrated with $0.05\text{ mol L}^{-1}\text{ HCl}$ after adding $1.0\text{ mol L}^{-1}\text{ BaCl}_2$ for precipitating carbonate at the incubation time of 10 h, 1, 3, 5 and 7 days using the approach proposed by You et al. (2014).

Measurements of soil enzyme activities

Two soil extracellular enzyme activities involved in degrading cellulose (β -1,4-glucosidase, BG, EC: 3.2.1.21) and chitin and peptidoglycan (β -1,4-N-acetyl-glucosaminidase, NAG, EC: 3.2.1.14), respectively, were measured. The activities of BG and NAG were determined on the basis of *p*-nitrophenol (PNP) released after cleavage of enzyme-specific synthetic substrates (You et al. 2014). In brief, the reaction mixture was composed of 0.2 mL 50 mM *p*-nitrophenyl- β -D-glucopyranoside and 0.2 mL 10 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide with 0.8 mL of shaken and filtered soil slurry (4 g fresh soil with 10 mL 50 mM sodium acetate buffer, pH 5.0) incubated in a dark microcosm at 37 °C for 1 h. BG and NAG activities were measured with ultraviolet spectrophotometer at 410 nm after filtration, respectively. The total enzyme activities were expressed as $\mu\text{mol g}^{-1} \text{h}^{-1}$, on soil dry weight basis.

Calculation of microbial biomass-specific respiration and carbon use efficiency

Based on the measurements of MBC and MR, the microbial biomass-specific respiration ($q\text{CO}_2$) was calculated as (You et al. 2014):

$$q\text{CO}_2 = \text{MR}/\text{MBC} \text{ (mg CO}_2 \text{ g}^{-1} \text{ C}_{\text{mic}} \text{ day}^{-1}\text{)}.$$

Microbial C use efficiency based on C:N stoichiometry ($\text{CUE}_{\text{C:N}}$) was calculated using the stoichiometric method proposed by Sinsabaugh et al. (2016) as:

$$\text{CUE}_{\text{C:N}} = \text{CUE}_{\text{MAX}}[\text{S}_{\text{C:N}}/(\text{S}_{\text{C:N}} + \text{K}_{\text{C}})], \text{ where } \text{S}_{\text{C:N}} = (1/\text{EEA}_{\text{C:N}})(\text{B}_{\text{C:N}}/\text{L}_{\text{C:N}}),$$

where $\text{S}_{\text{C:N}}$ is a scalar that represents the extent to which the allocation of enzyme activities offsets the disparity between the elemental composition of available resources and the composition of microbial biomass; K_{C} the half-saturation constant ($\text{K}_{\text{C}} = 0.5$); CUE_{MAX} the upper limit for microbial growth efficiency based on thermodynamic constraints, $\text{CUE}_{\text{MAX}} = 0.6$. EEA is the extracellular enzyme activity; $\text{EEA}_{\text{C:N}}$ was calculated as the ratio of BG to NAG (i.e., BG/NAG), where $\text{BG} = \beta$ -1,4-glucosidase and $\text{NAG} = \beta$ -1,4-N-acetyl-glucosaminidase. Molar ratio of SOC to TN was used as estimates of $\text{L}_{\text{C:N}}$.

Microbial biomass C:N ($\text{B}_{\text{C:N}}$) was also calculated as the molar ratio of microbial biomass C to N. The $\text{CUE}_{\text{C:N}}$ calculated here represents the community-level microbial CUE (Geyer et al. 2016).

Statistical analysis

A linear mixed-effects model was used to determine the effects of litter type treatments and N addition on physical and chemical fractions of SOC, MBC, MR, $q\text{CO}_2$, $\text{CUE}_{\text{C:N}}$ and enzyme activities. The litter type treatments and N addition, as well as the interaction between these two treatments were treated as fixed factors, and a plot nested within block term was treated as random factors. LSD test (least significant difference test) was used for post hoc multiple comparisons. The linear mixed-effects models were performed using the “lme4” package (Bates et al. 2014) in R 3.6.1 (R Development Core Team 2018). Prior to the analysis, the normality (Shapiro test) and the homoscedasticity of variance (Levene’s test) of all variables were tested, and data were log-transformed for variables not conforming to normality. We also calculated the Cohen’s *d* index of effect sizes of litter type treatments and N addition on soil organic carbon fractions and microbial properties. Pairwise comparisons of SOC fractions and their relationships with microbial characteristics were determined using Pearson’s correlation coefficient. Redundancy analysis (RDA) was used to analyze multivariate relationships between microbial characteristics and soil C and N properties. Forward selection (“ordistep” function) based on Monte Carlo tests with 999 permutations was used to identify key variables controlling microbial processes (i.e., MBC, MR, $\text{CUE}_{\text{C:N}}$, and $q\text{CO}_2$) using the package “Vegan” (Oksanen et al. 2016).

Results

TOC and DOC

Litter treatments had significant effects on the contents of TOC and DOC across all the three soil layers ($p < 0.05$; Fig. 1). The effects of N addition treatments were much weaker compared with the litter treatments, and were only significant for TOC in the top two soil layers (Fig. 2). There was no significant interactive effect between litter and N treatments on

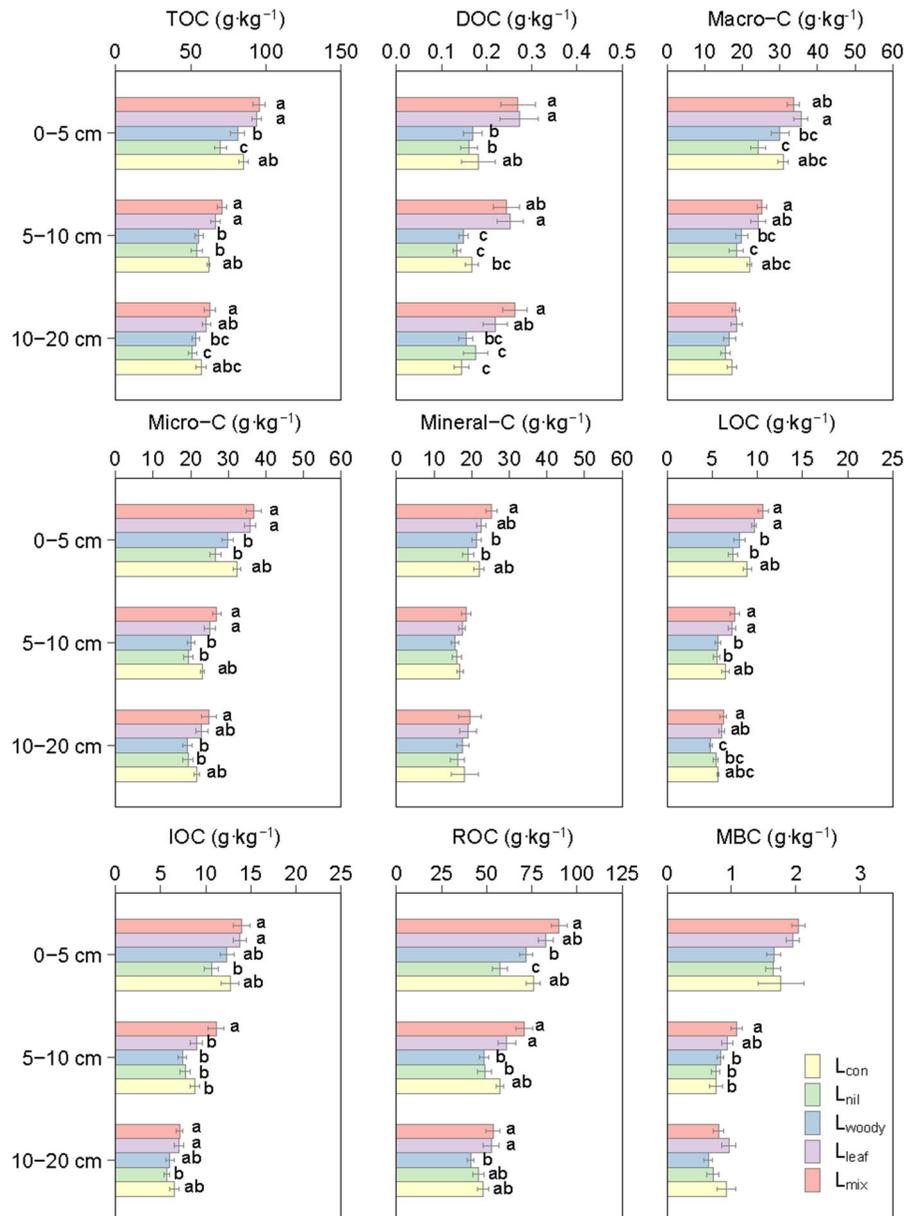


Fig. 1 Effects of litter manipulation on total soil organic carbon (TOC), dissolved organic carbon (DOC), three physical fractions and three chemical fractions of SOC, and microbial biomass carbon (MBC) in 0–5, 5–10 and 10–20 cm soil layers. Macro-C, soil organic carbon in the 250–2000 μm soil aggregates; Micro-C, soil organic carbon in the 53–250 μm soil aggregates; Mineral-C, soil organic carbon in the < 53 μm soil aggregates. LOC labile organic carbon; IOC intermediate

organic carbon; ROC recalcitrant organic carbon. *L_{con}* natural control soils; *L_{nil}* complete removal of all aboveground litter; *L_{woody}* placement of fine woody litter; *L_{leaf}* placement of leaf litter; *L_{mix}* placement of mixed leaf and fine woody litter. Error bars show one standard error. Different letters above the bar for the same soil layer indicate significant difference among treatments ($p < 0.05$)

TOC and DOC in any of the three soil layers ($p > 0.05$). An apparent phenomenon was that both TOC and DOC under *L_{nil}* and *L_{woody}* (treatments

without leaf litter) were significantly lower than that under *L_{leaf}* and *L_{mix}* (treatments with leaf litter) in three soil layer depths (Fig. 1). However, both TOC

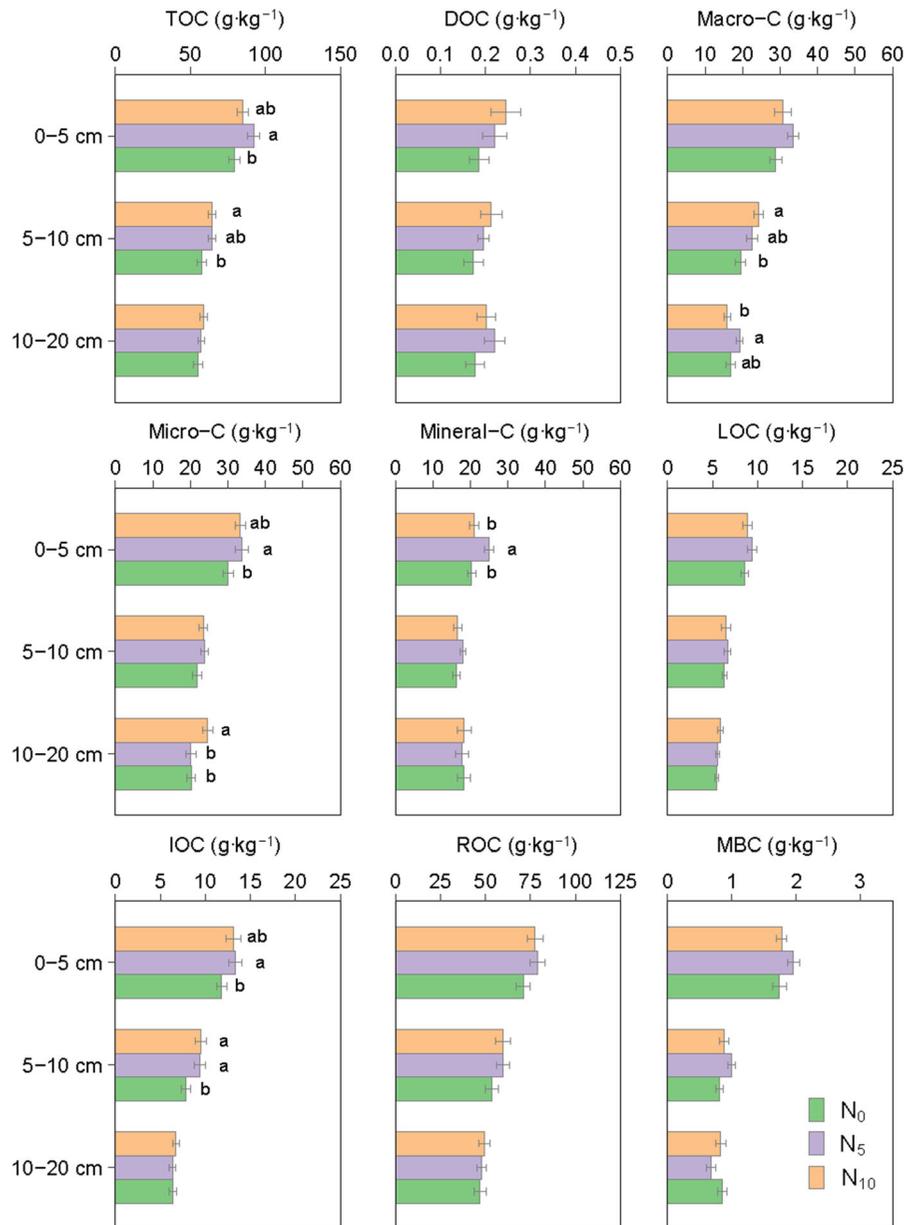


Fig. 2 Effects of nitrogen addition on TOC, DOC, Macro-C, Micro-C, Mineral-C, LOC, IOC, ROC and MBC in 0–5, 5–10 and 10–20 cm soil layers. N_0 , 0 g N m⁻² year⁻¹; N_5 , 5 g N m⁻² year⁻¹; N_{10} , 10 g N m⁻² year⁻¹ applied as urea.

Error bars show one standard error. Different letters above the bar for the same soil layer indicate significant difference among treatments ($p < 0.05$)

and DOC did not significantly change under the four litter treatments compared to L_{con} (Fig. 1) except that TOC under L_{nil} was significantly 17.9% lower in the 0–5 cm soil layer, DOC under L_{leaf} significantly 34.2% higher in the 5–10 soil layer, and DOC under L_{leaf} and L_{mix} significantly 34.4% and 45.3% higher in

the 10–20 cm soil layer, respectively. Moreover, the effect sizes of litter and N treatments on TOC and DOC were similar to that of the linear mixed-effects model in the three soil layers except that the TOC in the 0–5 and 5–10 cm soil layers and DOC in the 5–10 cm soil layer were significant lower under L_{nil}

than under the L_{con} (Figs. S1–S3). N addition treatments resulted in significantly higher TOC under N_5 in the 0–5 cm soil layer according to the effect size (Figs. S1–S3). The proportion of DOC in TOC, fDOC, in the 0–5 cm and 5–10 cm soil layers were significantly affected by litter treatments, with much lower fDOC under L_{woody} and L_{nil} compared with that under L_{leaf} ($p < 0.05$; Table S1). Significant interactions between litter and N treatments on fDOC occurred in the 0–5 cm and 10–20 cm soil layers, such that fDOC was much higher under L_{leaf} and L_{mix} than that under L_{woody} ($p < 0.05$; Table S1). Compared to L_{con} , none of the litter and N treatments significantly altered fDOC ($p > 0.05$; Table S1).

Physical and chemical fractions of SOC

The effects of litter and N treatments on the contents of physical SOC fractions varied with the fraction types and differed among the soil layers. Significant interactions between the litter and N treatments were only observed for the Macro-C fractions in the 10–20 cm soil layer ($p < 0.05$; Table S1). On average, the contents of all the three physical fractions (Macro-C, Micro-C, and Mineral-C) were greater under treatments with presence of leaf litter (i.e., L_{leaf} and L_{mix}) than those without (i.e., L_{nil} and L_{woody}), especially in the top 0–5 cm soil layer (Fig. 1). The contents of Macro-C and Micro-C in the 5–10 cm soil layer and Micro-C in the 10–20 cm soil layer were significantly greater under the litter treatments of L_{leaf} and L_{mix} than under L_{nil} and L_{woody} . However, all the three physical SOC fractions under the four litter treatments did not show significant differences compared to that under L_{con} in all the three soil layers (Fig. 1). With consideration of the effect size, the Macro-C in the 0–5 cm soil layer and Micro-C in the 0–5 and 5–10 cm soil layers under L_{nil} were significant lower compared to the L_{con} (Figs. S1, S2). In terms of the relative changes to L_{con} , they ranged from –28 to 14% for all treatments and in all soil layers.

N addition treatments significantly affected the contents of Micro-C and Mineral-C in the 0–5 cm soil layer, Macro-C in the 5–10 cm soil layer, and Macro-C and Micro-C in the 10–20 cm soil layer (Fig. 2), which were consistent with the results of the effect sizes (Figs. S1–S3). The contents of the three physical fractions were not significantly different between N_5 and N_{10} . Litter treatments had no significant effects on

fMacroC, fMicroC and fMineralC in all the three soil layers (Fig. S7); whilst N addition treatments significantly affected fMacro-C and fMicro-C in the 10–20 cm soil layer (Fig. S8). On average, 36%, 38% and 26% of SOC were stored in Macro-, Micro-, and Mineral-aggregates in the 0–5 cm soil layer respectively, and 35%, 37% and 28% in the 5–10 cm soil layer respectively, and 31%, 38% and 31% in the 10–20 cm soil layer respectively.

The contents of chemical SOC fractions (i.e., LOC, IOC and ROC) were significantly affected by litter treatments in all soil layers (Fig. 1). The effects of litter treatments on the chemical SOC fractions were similar to their effects on physical SOC fractions. Notably, the three chemical SOC fractions were all significantly greater under L_{mix} than under L_{nil} (Fig. 1). Compared to L_{con} , IOC under the L_{mix} was significantly 21.0% greater in the 5–10 cm soil layer and ROC under the L_{nil} significantly 24.2% smaller in the 0–5 cm soil layer (Fig. 1). The size analysis also showed the LOC in the 0–5 cm soil layer, IOC in the 10–20 cm soil layer and ROC in the 0–5 and 5–10 cm soil layers were significantly higher under L_{mix} than that under L_{con} (Figs. S1–S3). The effect of N addition treatments was only significant on the content of IOC in the 0–5 cm and 5–10 cm soil layers (Fig. 2). Significant interactive effects between litter and N treatments were observed on LOC and ROC in the 10–20 cm soil layer (Table S1). In terms of the relative quantity (%) of the chemical fractions, they were not significantly affected by the litter and N treatments. Consistently, ROC accounted for the majority (> 78%) of the SOC in the three soil layers.

Microbial biomass, respiration, carbon use efficiency and enzymatic activities

Overall, the treatment responses of the microbial characteristics investigated (i.e., MBC, $CUE_{C:N}$ and qCO_2) were mostly insignificant across three soil layers except for microbial respiration (i.e., MR). Only MBC in the 5–10 cm soil layer was significantly greater under L_{mix} than under L_{con} , L_{nil} and L_{woody} (Fig. 1). A significant interactive effect between litter and N treatments on MR occurred in the 0–5 cm soil layer (Table S1). While the effects of litter treatments on MR were significant in all three layers (Fig. 3), the effects of N addition treatments were only significant in the 0–5 cm and 5–10 cm soil layers (Fig. 4). MR

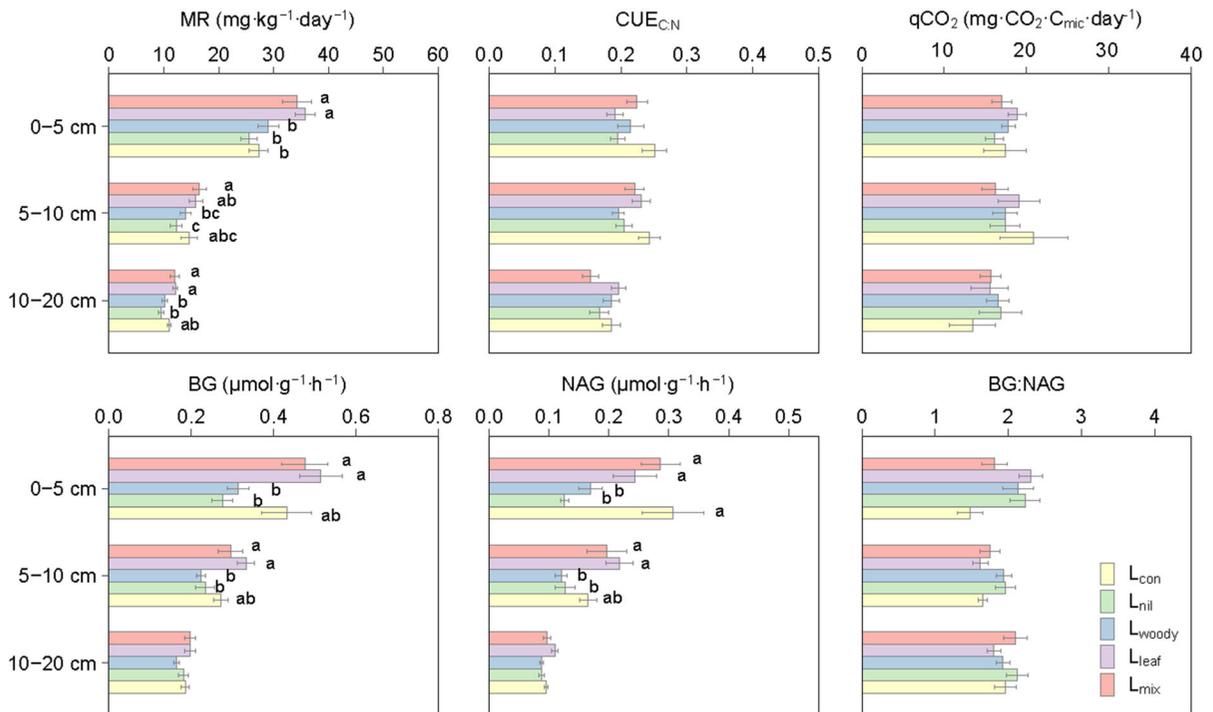


Fig. 3 Effects of litter manipulation on soil microbial properties (i.e., MR, $CUE_{C:N}$, qCO_2 , BG, NAG and BG:NAG) in 0–5, 5–10 and 10–20 cm soil layers. MR, microbial respiration; $CUE_{C:N}$, carbon use efficiency; qCO_2 , microbial

was 20.3% and 23.5% greater ($p < 0.05$) under L_{mix} and L_{leaf} , respectively, than under L_{con} in the 0–5 cm soil layer (Fig. 3). There was no significant effect of litter treatments on $CUE_{C:N}$ and qCO_2 in any of the three soil layers ($p > 0.05$, Fig. 3). In consideration of the effect size, however, $CUE_{C:N}$ under L_{leaf} in the 0–5 cm soil layer and under L_{nil} and L_{woody} in the 5–10 cm soil layer were significantly lower than that under L_{con} (Figs. S4, S5). The activities of two extracellular enzymes—BG and NAG—were significantly greater under L_{leaf} and L_{mix} than under L_{nil} and L_{woody} in the 0–5 cm and 5–10 cm soil layers (Fig. 3). NAG was 44.6% and 59.0% lower under L_{woody} and L_{nil} , respectively, than under L_{con} in the 0–5 cm soil layer. The effects of N addition treatments and its interaction with litter treatment on enzyme activities, $CUE_{C:N}$ and qCO_2 were insignificant in the three soil layers ($p > 0.05$; Fig. 4). The ratio of BG to NAG exhibited no significant response to litter and N treatments in all soil layers except for the effects of N addition in the 10–20 cm soil layer (Figs. 3 and 4). However, the ratio of BG to NAG was significantly

metabolic quotient; BG, β -1,4-glucosidase; NAG, β -1,4-N-acetyl-glucosaminidase. Error bars show one standard error. Different letters above the bar for the same soil layer indicate significant difference among treatments ($p < 0.05$)

higher under L_{nil} , L_{woody} and L_{leaf} in the 0–5 cm soil layer and under L_{nil} and L_{woody} in the 5–10 cm soil layer than under the L_{con} , and significantly lower in the 10–20 cm soil layer under N_{10} than under N_0 , based on the assessment by effect size (Figs. S4–S6).

The association of microbial properties with SOC compositions and nutrient status

MBC, MBN and MR were all positively correlated with soil physical and chemical SOC fractions in the 0–5 cm and 5–10 cm soil layers (Fig. 5). There were significant negative correlations of MBC and MBN with fIOC in the 0–5 cm soil layer and between MR and fMineral-C in the 5–10 cm soil layer. $CUE_{C:N}$ was not significantly correlated with either SOC fractions or the N addition level in all three soil layers (Fig. 5). qCO_2 was positively correlated with DOC, DOC/TOC and DON in the 0–5 cm soil layer and with NO_3^- -N and fROC in 10–20 cm soil layer (Fig. 5).

According to the RDA, in the 0–5 cm soil layer, the assessed seven SOC component fractions and N

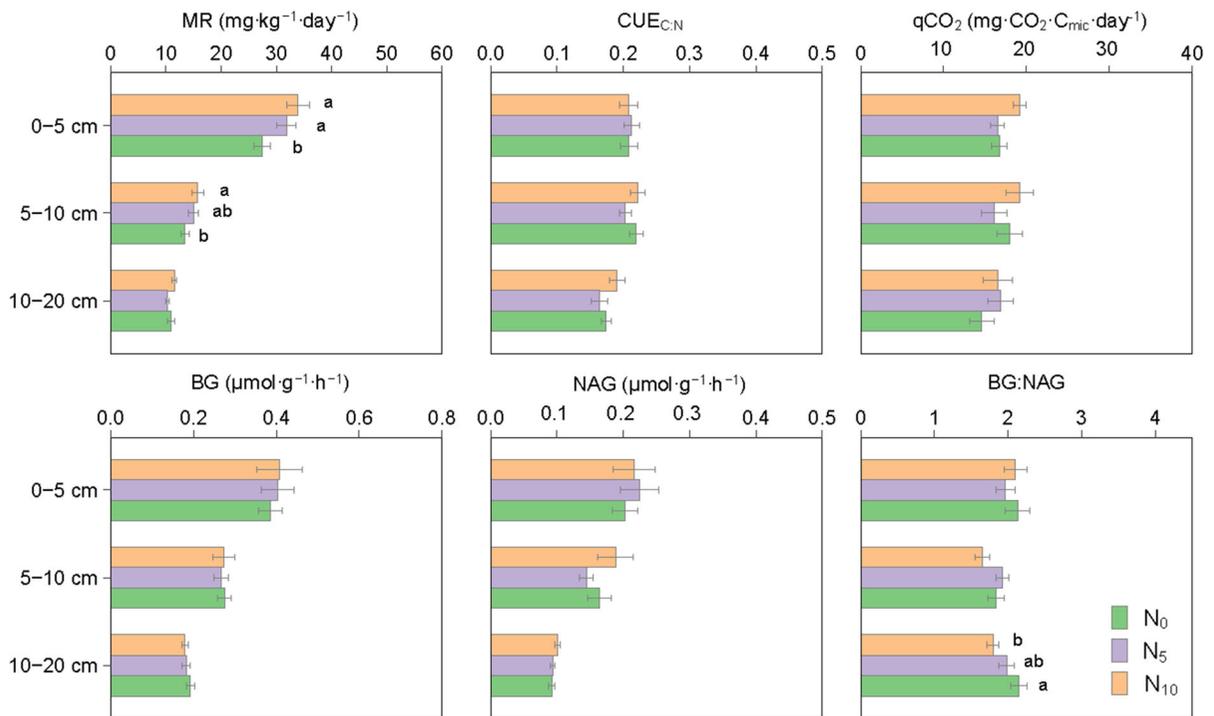


Fig. 4 Effects of nitrogen addition on soil microbial properties (i.e., MR, CUE_{C:N}, qCO₂, BG, NAG and BG:NAG) in 0–5, 5–10 and 10–20 cm soil layers. Error bars show one standard error.

Significant differences in the same soil layer are denoted by different letters ($p < 0.05$)

addition level accounted for 61% of the total variance in MBC, MR, CUE_{C:N} and qCO₂ (Fig. S9). Particularly, RDA identified that LOC, DON and DOC/TOC were the strongest predictors for MBC, MR, and qCO₂, respectively (Fig. 6). In the 5–10 cm soil layer, RDA explained 49% of the variance in microbial properties investigated (Fig. S9); TN and DOC were the most influential variables on MBC and MR, respectively (Fig. 6). In the 10–20 cm soil layer, 57% of the total variation in microbial characteristics was explained by the RDA (Fig. S9); and NH₄⁺-N, IOC and NO₃⁻-N were the most important variables affecting MBC, MR and qCO₂ (Fig. 6). Specifically, MBC, MR and qCO₂ were significantly and positively correlated with the most critical predictors identified in three soil layers.

Discussion

Effects of litter treatments on SOC fractions

Inconsistent with our first hypothesis, we found that the litter type alone played a predominant role in

controlling the content in different fractions of SOC. Changes in SOC were mainly related to the litter inputs of relatively high quality (i.e., leaves in this study) rather than the quantity. There were three lines of evidence. First, almost all physical and chemical C fractions were significantly higher under L_{leaf} and L_{mix} (the treatments with leaves) than under L_{woody} (the treatment without leaves). Secondly, there were no significant differences between L_{leaf} and L_{mix} in the total SOC and its composition. As the main difference between L_{leaf} and L_{mix} is that L_{leaf} includes leaf litter only, while L_{mix} includes woody and leaf litter, the similar effects of L_{leaf} and L_{mix} on SOC and its composition indicate that woody component has very limited influence after eight years of treatment. Thirdly and more directly, total SOC and its composition were comparable between L_{nil} (the treatment without any forest-floor litter input) and L_{woody}, suggesting that woody litter does not significantly contribute to the SOC pool, at least in the duration of eight years in our study.

This dominant effect of litter quality may attribute to that the decomposer community preferentially

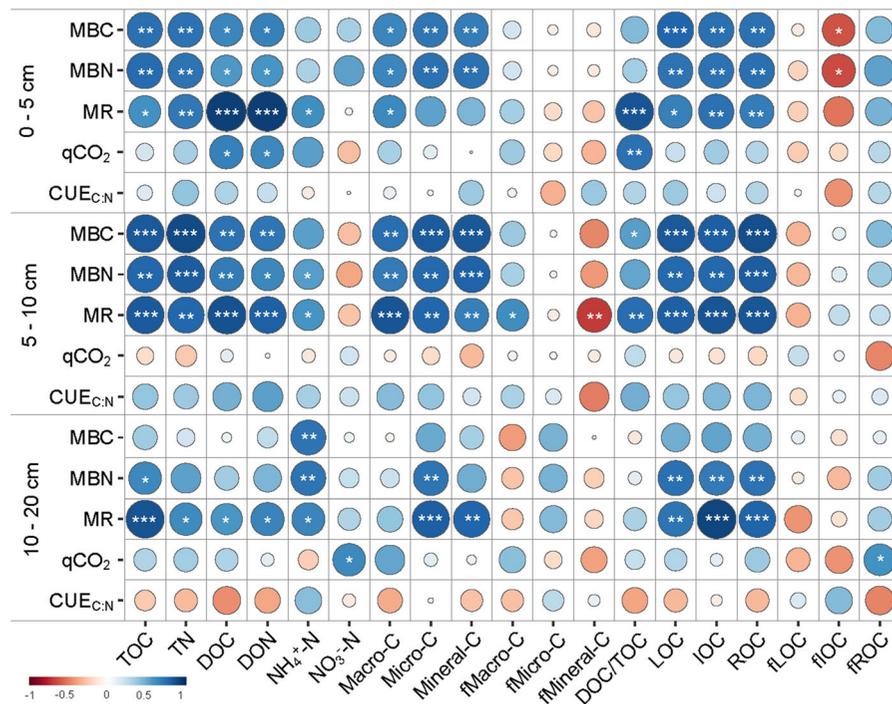


Fig. 5 Pearson's correlation coefficients matrix between soil microbial properties and SOC composition and nutrient status is displayed with a color gradient in 0–5 cm, 5–10 cm and 10–20 cm soil layers. Blue indicates positive correlation; red indicates the negative correlation. The darker the color, the greater the correlation. fMacro-C, the proportion of OC in the 250–2000 μm soil aggregates; fMicro-C, the proportion of OC

in the 53–250 μm soil aggregates; fMineral-C, the proportion of OC in the < 53 μm soil aggregates. fLOC, the proportion of labile organic carbon; fIOC, the proportion of intermediate organic carbon; fROC, the proportion of recalcitrant organic carbon. The asterisk denotes the statistical significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (Color figure online)

fragment/decay high-quality litter (Hättenschwiler et al. 2005), resulting in quick evolution of high-quality litter to small particles including microbial debris, which again are easier to be translocated to the mineral soil (Moorhead and Sinsabaugh 2006). In addition, high-quality litter contains a large fraction of dissolvable organic materials which directly contribute to mineral SOC pool through percolating water (Kalbitz et al. 2000). Indeed, this point was supported by the significantly higher DOC under L_{leaf} and L_{mix} (Fig. 1). Despite that L_{leaf} and L_{mix} significantly increased the soil physical and chemical C fractions compared with L_{nil} , no significant differences were found compared with L_{con} (Fig. 1), which may be due to soil priming induced under L_{leaf} and L_{mix} that compensates for new inputs of organic C (Lajtha et al. 2018; Sayer et al. 2011). Indeed, L_{leaf} and L_{mix} resulted in significantly higher enzyme activities and microbial respiration than L_{woody} and L_{nil} in the 0–5 and 5–10 cm soil layers (Fig. 3), which implies the

existence of the positive priming effects (Sayer et al. 2011; Cotrufo et al. 2013).

Effects of N addition on SOC fractions

Compared with the general significant main effects of litter treatment on all SOC fractions in all soil layers, the effects of N addition were relatively limited. We only detected significant effects of N addition treatment on total SOC and several specific SOC fractions in selective soil layers. For example, N addition treatments significantly increased total SOC in the 0–5 and 5–10 cm soil layers (Fig. 2). A major reason could be that many temperate forests are N-limited (Nadelhoffer et al. 1999), which resulted in limited or no change in the formation and transformation of SOC with additional N inputs. This is also supported by the results of microbial enzyme activity involved in N acquisition. That is, N addition did not significantly affect the activity of NAG in all three soil layers in this

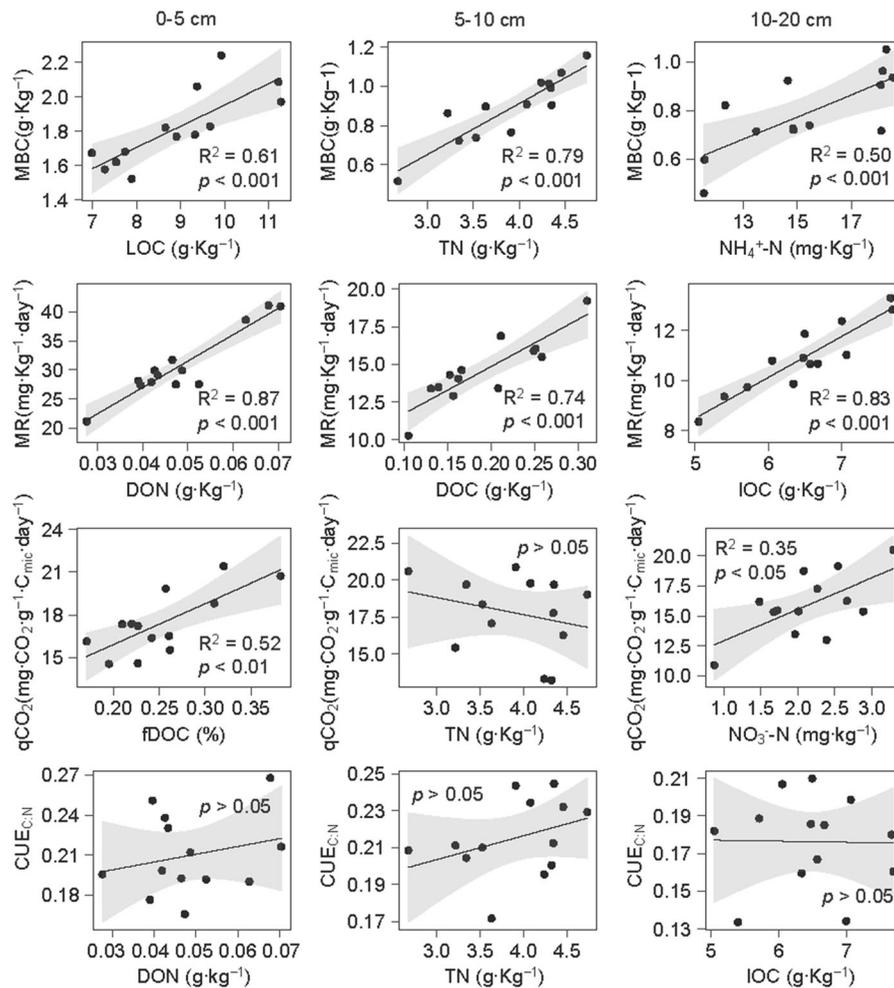


Fig. 6 The relationship between microbial properties (i.e., MBC, MR, CUE_{C:N}, and qCO₂) and their corresponding most important predictors identified by redundancy analysis (RDA) in

the 0–5 cm, 5–10 cm and 10–20 cm soil layers. TN total nitrogen; DOC, DON dissolved organic carbon and nitrogen

study (Fig. 4). Previous investigations in this experiment also found that the activities of BG, NAG, phenol oxidase and peroxidase were not affected by five years of N addition in the 0–5 cm soil layer (Sun et al. 2016).

Janssens et al. (2010) had proposed two, mutually non-exclusive, mechanisms to explain the N-induced response of SOC cycling: (1) enhanced chemical stabilization of organic C into recalcitrant compounds which are resistant to microbial decay; and (2) preferential utilization of labile, energy-rich compounds by microbes, but retarded decomposition of recalcitrant compounds. Based on our data, nonetheless, we did not find solid evidence to support these two hypotheses because none of the three chemical

fractions showed significant difference among the N addition levels except for IOC in the 0–5 and 5–10 cm soil layers (Fig. 2). Due to the slow formation of recalcitrant SOC compounds or saturation of mineral binding capacity, longer term experiment is required to detect changes in recalcitrant SOC pools to verify the two hypotheses. Moreover, Craine et al. (2007) found that microbes use labile substrates to acquire N from recalcitrant organic matter under low N availability and this “microbial nitrogen mining” is consistently suppressed by high soil N supply or substrate N concentrations. Lack of apparent treatment effects by N addition in this study could result from the possible resilience of the soil and microbial

communities to the relatively low level of N addition rate applied.

Different responses of physical and chemical fractions to litter and N treatments

The physical protection of SOC from decomposition (e.g. SOC bonds with silt and clay aggregates to form organic-mineral complex) and the chemical decomposability of SOC components are two important mechanisms underpinning SOC stabilization (Schmidt et al. 2011; Hemingway et al. 2019). In this study, litter treatment did not significantly increase the mineral-bound organic C fractions (fMineral-C) in different soil layers (Fig. S7), but the L_{woody} and L_{mix} (the treatments with woody litter) significantly increased fROC compared with L_{nil} in the 0–5 cm soil layer (Fig. S7). It is likely that the input of relatively recalcitrant woody litter significantly increased the proportion of chemically stable carbon components (e.g. lignin), and played a small role in regulating physical protection of SOC. However, all the four litter treatments did not significantly change fROC compared to L_{con} (Fig. S7), suggesting the weak influence of woody litter on SOC fractions. The very slow incorporation of woody litter to the mineral soil is likely the underlying reason for such insignificant effects even after eight years of experiment.

N addition treatments only had significant effects on one of the three chemical fractions (i.e., IOC) in the 0–5 cm and 5–10 cm soil layers, and its effect on the three physical SOC fractions were layer-dependent (Fig. 2). This result may be explained by N-induced changes in root growth and associated microbial processes (e.g. fungal hyphae), which are two main biotic factors regulating the formation and stability of soil aggregates (Miller and Jastrow 2000). For the formation and turnover of soil aggregates, most studies focused on agricultural soils (Six and Lenders 2000). Few studies have assessed the potential changes of soil aggregation processes under the change of litter and N inputs and the relevant consequences on SOC dynamics in natural ecosystems such as temperate forests. As SOC in different soil aggregates exhibits distinct turnover behaviors (von Lützow et al. 2007), the observed significant effects of N addition treatments on SOC in different aggregates offer an alternative mechanism to explain the observed

differential changes in SOC and its physical and chemical composition under N deposition.

Effects of litter and N treatments on soil microbial metabolism

There were significant positive correlations between SOC fractions (e.g., TOC, TN, DOC and DON) and microbial properties (e.g., MBC, MBN and MR) (Fig. 5), indicating the litter input may increase the microbial C substrate availability. Meta-analysis also found that litter inputs had a strong positive impact on soil respiration, labile C availability, and the abundance of soil microorganisms (Zhang et al. 2020). However, contrary to our second hypothesis that microbes adjust substrate utilization strategies under N addition and litter manipulation treatments, we found that microbial substrate utilization strategies are relatively persistent under the given treatments in this study. The two measures of microbial C use efficiency (i.e., $CUE_{C:N}$ and qCO_2) did not show significant response to either litter or N treatments (Figs. 3 and 4), albeit that the microbial respiration had been significantly affected by litter treatments in all soil layers (Fig. 3). In addition, the correlation analysis also showed that the magnitude of the correlation of $CUE_{C:N}$ and qCO_2 with variables reflecting soil substrate and nutrient status was lower than the magnitude of the correlation of $CUE_{C:N}$ and qCO_2 with microbial biomass and respiration (Fig. 5). These results may suggest that the energy and nutrient in the top 20 cm mineral soil layer at the studied site are not strong regulators and/or limiting factors of microbes; therefore microbes do not need to significantly adjust their functional properties such as C use efficiency (van Groenigen et al. 2013).

Several mechanisms may explain why microbial substrate utilization was unaffected by the imposed changes in N availability and litter inputs. Firstly, most energy and nutrient sources are inaccessible for microbes in the bulk soil environment (Schmidt et al. 2011). A number of studies have demonstrated that the accessibility of SOC (i.e., substrate availability) by microbes is the limiting factor of soil organic matter decomposition (Cotrufo et al. 2013; Dungait et al. 2012). For this reason, microbial activity and function would be determined by substrates that are readily available, which are also determined by the soil physical environment (e.g., microbial substrate

availability regulated by changes in soil temperature and moisture). Rather, both N addition treatments and litter manipulation in this study may have limited effects on the physical accessibility of SOC to microorganisms (Moinet et al. 2018). On the one hand, the added N may leave the system quickly due to the volatility of urea (Ramirez et al. 2010). On the other hand, as discussed above, the studied forest is not N-limited. In addition, more time may be required to degrade the added litter (especially woody litter) before microbial utilization. Secondly, microbial activity and substrate use efficiency would be limited by other environmental factors rather than substrate availability. For example, the effect of substrate availability on $CUE_{C:N}$ depends on the availability of other nutrients (e.g. phosphorus) and the elemental stoichiometry (Hessen et al. 2004; Sinsabaugh et al. 2013).

Soil moisture and temperature are also important environmental factors regulating microbial metabolism (Manzoni et al. 2012). In this study, compared with L_{nil} and L_{con} , increased litter cover (i.e., L_{mix}) significantly increased soil mass water content ($p < 0.05$); and there were also significant differences in soil temperature under the litter treatments ($p < 0.001$; Table S1). Meanwhile, all microbial properties were measured based on soil cores sampled at a particular point in time. As such, the measured microbial properties may only reflect the behavior of microbes at that particular time; while litter and N addition usually have a transient effect on microbial substrate use efficiency (Poehlau et al. 2019). It is important to monitor temporal dynamics of microbial processes in order to explicitly quantify the association of microbial properties with nutrient and energy availability.

Extracellular enzymes play a critical role in breaking down polymers into smaller molecules that can be directly used by microbes (Carreiro et al. 2000). Results in this study suggested that the two extracellular enzymes degrading cellulose (BG) and chitin and peptidoglycan (NAG) had significantly greater activities under L_{leaf} and L_{mix} than under L_{nil} and L_{woody} (Figs. 3 and S3–S6). It is especially important to note that their changes are nearly synchronous, i.e., the BG/NAG ratio was relatively constant under the litter and N treatments (Figs. 3 and 4). As two enzymes specifically metabolize different substrates (i.e., BG for C and NAG for N), the rate of increase in the

activity of one enzyme would override the rate of increase in the activity of the other if microbes are limited by C (or N). Their synchronous responses provide further evidence that litter manipulation and N addition do not shift the substrate (C) utilization strategy of microbes.

Conclusions

After 8 years of litter and N addition treatments in a mixed pine-oak forest under temperate climate, we found significant changes in SOC content including DOC and various physical and chemical component fractions. This change was predominantly controlled by the quality of carbon inputs represented by woody materials and leaves in this study. Compared to the control, however, the removal of forest-floor litter or increased inputs did not significantly affect the content in various SOC fractions after eight years, which might be due to reduced and stimulated decomposition under litter removal and input, respectively, resulting from soil priming. In order to quantify the contribution of aboveground litter components to mineral SOC pool, we strongly recommend that the dynamics of aboveground litter derived C pool and root-derived C should be properly traced (e.g. using C isotopes) and linked with the dynamics of mineral SOC pool. N input (i.e., in the form of urea in this study) could not compensate the effect of litter quality, as demonstrated by the general insignificant effect of either N addition or its interaction with litter input. This may be due to that the fertilization effect of N addition was weak or the urea-N addition leaves the system quickly via various pathways (e.g., leaching and volatilization). Although significant changes occurred in SOC fractions, microbial substrate utilization was relatively resistant to litter manipulation and N addition treatments in this study. Overall, our study demonstrates that litter quality change is a more significant regulator of SOC dynamics than litter quantity and soil N availability in this temperate forest.

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Author contributions OJS designed the field experiment. OJS and ZL conceived the study and interpreted the results. XG conducted measurements, performed field sampling and data analyses, and prepared the initial draft of the manuscript. ZL and OJS substantially revised the manuscript.

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