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Non-additive effects of nitrogen and phosphorus fertilization on microbial biomass and residue distribution in a subtropical plantation

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ABSTRACT

High nitrogen (N) and phosphorus (P) availability has significant influence on microbial-driven soil carbon (C) sequestration. Microbial residues are a significant contributor of soil stable C pool, their distribution among aggregate fractions determines long-term soil C stability. However, very little is known about the interactive effects of N and P fertilization on soil microbes, especially their residues, at aggregate scale in plantation ecosystems. Since 2012, a field-manipulated experiment with N (200 kg N ha^{-1} $year^{-1}$) and/or P fertilization (50 kg P ha^{-1} $year^{-1}$) has been conducted to examine their interactive effects on microbial community and residues in bulk soil and three soil aggregate fractions: large macroaggregates (>2 mm, LMA), small macroaggregates (0.25–2 mm, SMA), and microaggregates (<0.25 mm, MA) in a subtropical Chinese fir (*Cunninghamia lanceolata*) plantation. Results showed that N and P fertilization, either individually or in combination, decreased microbial biomass of bulk soils to a similar extent (by up to 37.0%). This reduction was due to the decreased bacterial biomass in SMA and MA and fungi in LMA. By contrast, adding N and P fertilizer together (NP) significantly stimulated fungal residues in SMA and further redistributed microbial residues from LMA to SMA, although single fertilization had no effects on microbial residues or their distribution. Changes in root biomass moderated the direct effects of fertilization on aggregate-associated microbial groups and the indirect effects of NP fertilization on microbial residue distribution. Together, our results provide new insights into the microbial mechanisms through which multiple fertilization control soil C persistence in subtropical plantation. These findings highlight that separating bulk soil into distinct aggregate fractions and considering the interactive effect of N and P fertilization are needed to predict the soil C dynamics under fertilization.

1. Introduction

Fertilization is an important silvicultural management practice to stimulate plant growth, and the stimulation is notably greater when adding mineral nitrogen (N) and phosphorus (P) fertilizer together [15,19]. Soil organic carbon (SOC) in forest ecosystems, accounting for more than 70% of global SOC [37], determines long-term ecosystem productivity and contributes to climate change mitigation [36,41]. However, compared with the relatively consistent response of plant productivity to fertilization, SOC response, especially to combined N and P fertilizers (NP), remains largely unknown in forest ecosystems (reviewed in Fang et al. [11]). Given that ecosystems almost always simultaneously experience inputs of N and P [31] and results from single-fertilizer experiments poorly predict the interactive effects of N and P [7], it is essential to understand how N interacts with P fertilizer to affect SOC sequestration to improve the integrated nutrient management of forest ecosystems.

SOC sequestration is mainly associated with the catabolic and anabolic activity of microbes [20]. Living microbes decrease SOC sequestration via mineralizing organic substrates into CO_2, but dead microbial biomass increase it via continuously accruing microbial residues in the relatively slow-cycling carbon (C) pool [28,34]. Such residues are increasingly recognized as a major determinant of stable SOC, contributing up to 65% of SOC in forests [20,29,30]. Thus,

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simultaneously examining microbial biomass and residues can improve our mechanistic understanding of SOC response to fertilization. Numerous studies have suggested that N fertilizer generally decreases microbial biomass and fungi-to-bacteria (F/B) ratio determined by phospholipid fatty acid (PLFA) analyses via enhancing soil acidification or C limitation (reviewed in Treseder [43] and Zhou et al. [52]); P fertilizer stimulates soil microbial growth in subtropical forests because it alleviates microbial P limitation [17,20], but NP interaction has been reported to be additive (the combined effect is equal to the sum of individual effects) [24,26] or synergistic (the combined effect is greater than the sum of the individual effects respectively) [9]. Compared with living microbial biomass, microbial residues under fertilization have received very limited attention, despite these residues determine long-term SOC accumulation and stabilization [20]. Ma et al. [26] found that N- and P-only fertilizer stimulated microbial residues and their contribution to SOC, and their interaction was synergistic in a subtropical plantation. However, Ma et al. [24] showed that adding N and P individually or in combination had no effect on microbial residues or their contribution to SOC in a tropical forest. These divergent findings may be attributable to different experiment designs (e.g., experimental duration and applied nutrient levels), but are more likely due to the different linkages among plant, microbes, and soil properties. However, current studies on these microbial responses are mainly conducted on bulk soil with microbes that are hierarchically distributed within soil aggregates receiving less attention. Given that soil aggregates likely mediate microbial responses via governing the spatial heterogeneity of soil physicochemical characteristics [38,42] and the linkage between plant and microbes [45], studying the effects of fertilization on soil microbes at finer soil scales is essential to uncover the mechanisms underlying these observed microbial responses.

As the basic element of soil structure, soil aggregates can protect SOC including microbial residues against decomposition, increasing its stability [39]. In turn, microbial residues can help form or stabilize soil aggregates, showing a more persistent effect than those of living biomass [8]. Generally, macro-aggregates (>0.25 mm) are characterized by greater oxygen, water diffusion rate, and labile substrates fostering more fungi and fungal residual contribution to SOC [38,42,49]. These aggregates provide shorter protection due to their greater susceptibility to physical disruption than micro-aggregates (<0.25 mm) [14]. Therefore, microbial residue distribution in aggregates could have a significant impact on long-term SOC sequestration. For now, the only forest study found that P fertilization decreased microbial residues and their contribution to SOC due to enhanced recycling of microbial residues via increased activity of residue-decomposing enzymes, and the P effect was consistent among aggregate sizes [49]. However, the study did not evaluate how microbial residue distribution respond to N and P fertilization, despite the latter strongly influence soil aggregate structure [6,44]. Moreover, whether this observation is site- or ecosystem-specific remains unclear and needs further validation.

The Chinese fir (*Cunninghamia lanceolata*) is a fast-growing and commonly planted tree species on lateritic soils in subtropical regions, with a planting area accounting for approximately 18.2% of the total plantation area in China [27]. However, timber production in Chinese fir plantations has been progressively limited by the loss of soil fertility with subsequent recommendations for N and P fertilization [51]. However, microbial response to these fertilization at aggregate scale remains unknown in plantation ecosystems. Here, we conducted a six-year field experiment to explore how N and P fertilization, independently and interactively, influence microbial biomass and residues at bulk and aggregate levels in the soil of a Chinese fir plantation. Microbial residues are routinely quantified by amino sugar analysis, because amino sugars are primarily derived from dead microbial cells and are relatively biochemically resistant to decay [10,16,23]. We hypothesized that (1) P fertilizer would increase microbial biomass and their residues, and its positive effect will be greater under N fertilizer (i.e., synergistic interaction) because N fertilizer aggravates ecosystem P limitation [18]; (2) fertilization-induced changes in plant C allocation and soil chemical properties would preferentially alter microbial biomass and residues in macroaggregates, thus modifying the distribution of microbial residues. Altogether, we aimed to promote the mechanistic understanding of soil microbial responses to multiple fertilizers in subtropical plantations.

2. Materials and methods

2.1. Site description and experimental design

The study was conducted at the Huitong Experimental Station of Forest Ecology (26° 40′N, 109° 26′E), Chinese Academy of Sciences, Hunan Province in southern China. The site has a subtropical monsoon humid climate. The annual mean precipitation is approximately 1200 mm, of which 60–70% falls between April and August. The annual mean temperature is 16.5 °C, ranging from 1.9 °C in January to 29.0 °C in July. Soil is predominantly typical lateritic, identified as according to the USDA soil classification system.

A field fertilization manipulation experiment was conducted in a five-year-old pure *C. lanceolata* plantation in 2012 with 3 replicate blocks. In this experiment, a randomized block design was used with four treatments as follow: control (CT, no N or P), N fertilization (200 kg N ha^{-1}, N), P fertilization (50 kg P ha^{-1}, P), and N plus P fertilization (200 kg N ha^{-1} plus 50 kg P ha^{-1}, NP). A total of 12 plots, with the size of 10 m × 10 m each, were 80 m away from each other to avoid interference. For each fertilizer application, urea and/or KH_2PO_4 were dissolved in 30 L distilled water and sprayed onto the forest floor, using a backpack sprayer. The CT plots was applied the same amount of distilled water (30 L) without adding any fertilizer. The fertilizers were applied once a year in April.

2.2. Soil sampling and separation of soil aggregates

Soils were randomly collected in July 2018. Six intact soil cores (0–10 cm depth) were sampled from each plot and mixed homogeneously to form one composite sample. All roots in the composite sample were dried at 65 °C and weighed to determine fine root biomass. After visible plant residues were removed, each soil sample was sieved (<8 mm) in the laboratory, and then divided into two subsamples. One subsample was further sieved (<2 mm) as bulk soil, half of them was air-dried for analysis of soil physicochemical properties and the remain was stored at −20 °C for microbial analyses. The other subsamples was separated into three aggregate-size by a dry-sieving method [45], which can maintain microbial activity and minimize the disturbance of microbial community during soil fractionation. Briefly, soils (100 g) were put on a Retsch AS200 Control (Retsch Technology, Dusseldorf, Germany) with two sieves (0.25 and 2 mm) stacked on each other. After isolation, large macro-aggregates (LMA, > 2 mm), small macro-aggregates (SMA, 0.25–2 mm), and micro-aggregates (MA, < 0.25 mm) were respectively weighed and then air dried for future use. The recovery of soil after fractionation was 98%–99% on average.

2.3. Soil properties analyses

Soil C and total N (TN) were analyzed by a C/N analyzer (Elementar, Germany). Soil available P (AP) was determined following by molybdenum blue colorimetry [22]. Soil pH was determined in a soil water solution (1:2.5 w/v). Soil exchangeable cations ($Ca^{2+}$and Mg^{2+}) were measured using the ammonium acetate method.

2.4. Phospholipid fatty acids (PLFAs) analysis

Soil microbial communities were quantified by the PLFAs analysis as described by Bardgett et al. [2]. Briefly, 5 g of freeze dried soil was extracted using a chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v). The extracted PLFAs were purified by chromatography on silicic

acid columns. After mild alkaline methanolysis, the purified PLFAs were then analyzed with a gas chromatograph (Agilent 6850, Agilent Technologies, Santa Clara, CA, USA) and quantified with 19:0 (methyl nonadecanoate, C20H40O2) as the internal standard. Fatty acids including i14:0, i15:0, a15:0, i16:0, a17:0, i17:0 were considered as Gram-positive (G+) bacteria, 16:1w7c, cy-17:0, 18:1w7, cy19:0 were considered as Gram-negative (G-) bacteria. Bacterial PLFAs were calculated as the sum of G+ bacteria, G-bacteria, and general bacterial PLFAs (including 15:0, 16:0, 17:0, 18:0 and 20:0 PLFAs). The PLFA, 16:1w5c, was used for arbuscular mycorrhizal fungi (AMF); and the PLFAs, 16:1w5c, 18:1w9c, and 18:2w6c were used as fungi.

2.5. Microbial residue analysis

Microbial residue was assessed by amino sugars following the methods of Zhang and Amelung [50]. Briefly, air-dried soils (ca. 0.2 g) were incubated with 6 M HCl for 8 h at 105 °C, and then the solution was filtered, adjusted pH to 6.6–6.8, dried on a rotary evaporator, and centrifuged. The supernatant solution was dried on the rotary evaporator again, then the residues were dissolved with 5 mL methanol, centrifuged, transformed into a vial and dried using N_2 gas. To purified amino sugars, the vial was added with aldononitrile derivatives, extracted with acetic anhydride and dichloromethane, and removed excessive an hydride using 1 M HCl and deionized water. Lastly, sample was dried using N_2 gas and dissolved with ethyl acetate-hexane (1:1, v/v). Amino sugar derivatives were identified on a gas chromatograph (Agilent 7820A, Agilent Technologies) equipped with an HP-5 column (30 m × 0.25 mm × 0.25 μm). Glucosamine (GluN) is derived mainly from fungal chitin, but muramic acid (MurN) is solely synthesized by bacteria, hence fungal-derived C as a biomarker of fungal residues was calculated by removing bacterial-derived GluN from total GluN, assuming that GluN and MurN occur at a 2:1 M ratio in bacterial cells [10]. As a result, fungal-derived C (g kg^{-1}) = (mmol GlcN−2 × mmol MurN) × 179.2 × 9; bacterial-derived C was an index for bacterial residues, which was calculated by MurN (g kg^{-1}) × 45, where 179.2 is the molecular weight of GlcN, 9 and 45 are the conversion value of GluN and MurN to fungal residues and bacterial ones, respectively [10]. Total microbial residues were estimated as the sum of fungal- and bacterial-derived C. The percentage contribution of microbial residue contents (C_{MR}) in the *i*th size fractions to the bulk soil (CP_i, %) was computed based on [40]:

$$CPi = P_i \times CC_i \div CC$$

where P_i stands for the proportion of aggregates, CC_i represents the C_{MR} in aggregates at the *i*th size (mg kg^{-1}), while CC indicates the C_{MR} in bulk soil (mg kg^{-1}).

2.6. Statistical analysis

Two-way Analysis of Variance (ANOVA) was conducted to test the effects of N, P, and their interaction on microbial communities and their residues in bulk soil and each aggregate fraction. Whenever ANOVA yielded significant effects, Duncan test was performed to assess the differences among fertilization treatments or aggregate fractions at $P < 0.05$. Moreover, linear regression were performed using the"lm"function in R to estimate the correlations of aggregate-associated microbial groups with soil properties and root biomass. A structure equation modeling (SEM) was fitted by maximum likelihood estimation to gain a mechanistic understanding of how root biomass and soil aggregate structure followed by NP fertilization mediated alterations in fungal residues and their distribution. The chi-square test ($\chi 2$) was used to examine the overall goodness of fit for SEM. Non-significant $\chi 2$ test ($P > 0.05$) and $\chi 2$/df within 0–2 indicate the SEM is acceptable [33]. The SEM analyses was conducted using lavaan package in R 3.4.1. Other statistical analyses were performed using SPSS 16.0 (SPSS Inc. Chicago, IL, USA) software.

3. Results

3.1. Soil properties and root biomass

Fertilization did not influence SOC in bulk soils and among aggregate fractions ($P > 0.05$; Table 1 and Fig. S1a). However, P fertilization, alone and in combination with N fertilization, significantly enriched soil available P in bulk soil, with markedly higher value under NP plots (P: $P < 0.001$; N × P: $P = 0.014$; Table 1). N fertilization significantly decreased soil pH by 0.58 unit in bulk soil ($P = 0.026$; Table 1), and the decrease was consistent among different aggregate sizes (all $P \leq 0.001$; Fig. S1b). Moreover, N fertilization significantly suppressed Ca^{2+} and Mg^{2+} cations in bulk soil ($P = 0.006$; Table 1) by lowering them in large macroaggregates (LMA) and small macroaggregates (SMA) ($P = 0.007$ and 0.001, respectively; Fig. S1c). N and P fertilization significantly inhibited root biomass by 43.7% and 42.5%, respectively (both $P \leq 0.001$; Table 1), with the lowest value noted in NP plots. Meanwhile, N fertilization and its interaction with P fertilization significantly decreased the proportion of LMA (N: $P = 0.014$; N × P: $P = 0.016$) but increased that of SMA (N: $P = 0.002$; N × P: $P = 0.005$; Table 1).

3.2. Soil microbial communities

Adding N and P fertilizers, individually and in combination, decreased the PLFAs of bulk soils to a similar extent (by 27.9%–37.0%, P: $P = 0.037$; N × P: $P = 0.012$; Fig. 1a). The negative effects of fertilization on total PLFAs only occurred in SMA (N × P: $P = 0.024$) and microaggregates (MA) (P: $P = 0.023$; N × P: $P = 0.036$) but not in LMA (Fig. 2a). The responses of bacterial PLFAs in bulk soil and aggregate fractions mirrored those of total PLFAs (Figs. 1a and 2b). By contrast, N fertilization respectively reduced fungal and AMF PLFAs by 18.2% ($P = 0.003$) and 30.0% ($P = 0.023$) in bulk soils (Fig. 1a) by lessening them in LMA (both $P < 0.001$; Fig. 2c and d). P fertilization marginally suppressed fungal PLFAs in bulk soils ($P = 0.088$; Fig. 1a) but significantly reduced them by 10.7% in LMA ($P = 0.012$; Fig. 2c). Moreover, N fertilization significantly inhibited the F/B ratio in LMA, and its interaction with P fertilization influenced the ratio of Gram-positive to Gram-negative (G+/G-) bacteria in MA ($P = 0.002$ and 0.027, respectively; Fig. 2e and f). However, no significant effects were observed on such ratios in bulk soils (all $P > 0.05$; Fig. 1b).

3.3. Soil microbial residues

Fertilization did not affect microbial residues, their contribution to SOC, and the ratio of fungal to bacterial residues in bulk soils (all $P > 0.05$; Fig. S2). These non-significant effects also held true for aggregate-associated total microbial residues, bacterial residues, and microbial contribution to SOC across different aggregate fractions (all $P > 0.05$; Fig. 3a, b, and e). However, P fertilization significantly stimulated fungal residues in LMA and the ratio of fungal to bacterial residues in MA by 19.3% and 27.8%, respectively ($P = 0.033$ and 0.031, respectively; Fig. 3c and d). In addition, compared with other treatments, NP fertilization had higher fungal residues in SMA (N: $P = 0.012$, P: $P = 0.04$, N × P: $P = 0.009$; Fig. 3c). More importantly, NP fertilization significantly suppressed the contribution of total, bacterial and fungal residues in LMA by 26%–36.7% (N: $P = 0.001$; N × P: $P = 0.007$), 26.4%–31.2% (N × P: $P = 0.03$) and 20%–39.9% (N: $P < 0.001$; N × P: $P = 0.014$), respectively, but significantly stimulated that of total and fungal residues in SMA by 22.1%–37.5% (N: $P < 0.001$; N × P: $P = 0.07$) and 24.5%–29.4% (N: $P < 0.001$; N × P: $P = 0.003$), respectively (Fig. 4).

Table 1
Soil properties and root biomass in bulk soil under N, P, and NP fertilization.

Treatments	SOC(g kg^{-1})	TN (g kg^{-1})	AP (mg kg^{-1})	pH	Soil exchangeable Ca^{2+} and Mg^{2+} cations (mg/kg)	Root biomass (g m^{-2})	Aggregate mass proportion (%)		
							LMA	SMA	MA
CT	14.27 ± 1.2	1.19 ± 0.1	16.69 ± 2.5c	5.25 ± 0.1**a**	168.6 ± 10.2**a**	95.9 ± 4.2**a**	42.0 ± 2.8**a**	40.64 ± 1.5**b**	17.36 ± 2.1
N	12.47 ± 0.7	1.11 ± 0.1	13.57 ± 0.5c	4.95 ± 0.1**b**	139.2 ± 4.2 **ab**	62.1 ± 5.4**b**	41.84 ± 32.8**a**	41.32 ± 2.0**b**	16.84 ± 1.3
P	13.8 ± 0.9	1.19 ± 0.1	73.4 ± 1.9b	5.33 ± 0.1**a**	157.3 ± 4.5**a**	63.3 ± 1.8**b**	47.37 ± 2.2**a**	37.95 ± 1.4**b**	14.68 ± 0.9
NP	14.54 ± 0.9	1.23 ± 0.1	90.37 ± 6.7a	5.05 ± 0.1**b**	135.3 ± 6.9**b**	27.6 ± 2.3**c**	33.48 ± 1.9**b**	47.40 ± 1.6**a**	19.12 ± 0.8

Data are means ± SE (n = 3). Different letters within the same column indicate significant differences among nutrient treatments at $P < 0.05$. SOC, soil organic carbon; TN, total N; AP, available P; LMA, > 2 mm aggregate; SMA, 0.25–2 mm aggregate; MA, < 0.25 mm aggregate.

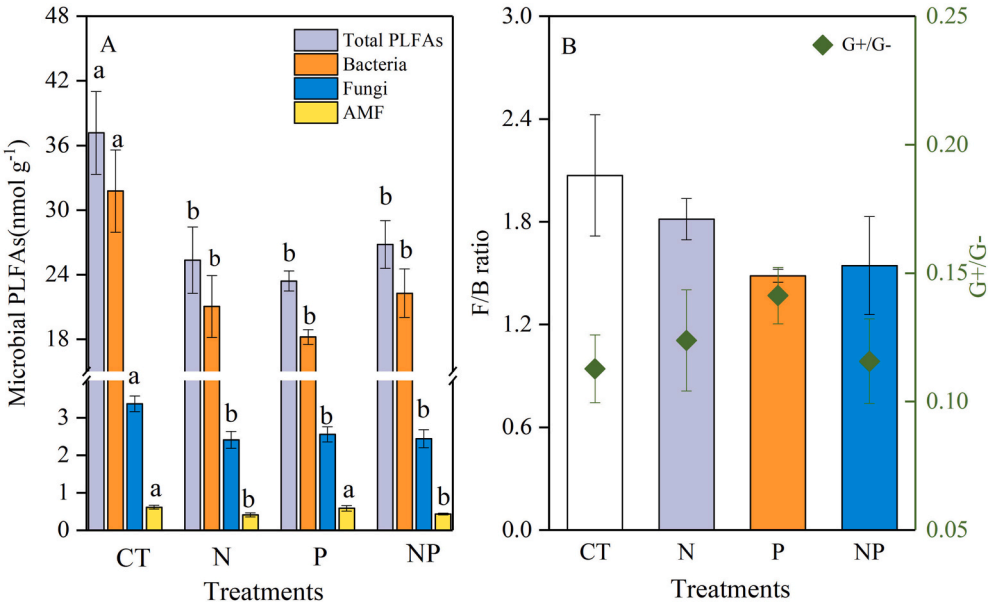

Fig. 1. Effects of N fertilization, P fertilization, and N plus P fertilization on microbial communities in bulk soils. Error bars indicate mean ± SE (n = 3). Different letters represent significant differences among nutrient treatments at $P < 0.05$. AMF, arbuscular mycorrhizal fungi; F/B ratio, fungi-to-bacteria; G+/G-ratio, Gram-positive to Gram-negative bacteria; PLFAs, phospholipid fatty acids.

3.4. Drivers of microbial groups and residues

The aggregate-associated microbial groups exhibited significantly positive correlations with root biomass (Fig. 5), but did not correlate well with soil chemical properties except for soil pH in LMA ($P = 0.016$, Table S1).

The structural equation models (SEM) showed that NP fertilization had a direct effect on the proportion of SMA (std. coefficients = 0.52, $P = 0.02$), which positively influenced the distribution of fungal residues in SMA (std. coefficients = 0.86, $P < 0.001$; Fig. 6). NP fertilization also had a positive indirect effect on fungal residues in SMA and a negative indirect effect on the distribution of fungal residues in LMA through root-driven aggregate breakdown (all $P < 0.001$). Overall, the SEM explained 76% of the variability in fungal residues in SMA, and 74% and 69% of the distributions of fungal residues in SMA and LMA, respectively (Fig. 6).

4. Discussion

4.1. Effects of fertilization on microbial biomass

It has been widely recognized that N fertilization decreases microbial biomass in bulk soils [23,43,47], which is mainly attributed to N-induced soil acidification and associated loss of base cations, especially Ca^{2+} and Mg^{2+} [43,47] and C limitation [52]. However, studies on bulk soils generally ignore soil aggregates which mediate the microbial response to N fertilization via affecting soil physical and chemical properties, and plant-soil linkages [45,49]. Therefore, understanding N-microbe linkages at finer soil scales is necessary to reveal the mechanism of N-induced decline in microbial biomass. In the present study, reduced microbial biomass with N fertilization was the consequence of lower bacteria in SMA and MA and fungi in LMA (Fig. 2b and c). The regression analysis clearly showed that these aggregate-associated microbial groups elevated linearly with root biomass (Fig. 5). Although positive correlation also occurred between fungi and soil pH in LMA (Table S1), consistent decline in the soil pH in other two aggregates with N fertilization did not cause a corresponding decrease in aggregate-associated fungi (Figs. 2 and S2b). Taken together, our findings provide strong evidence that N-induced declines in root C input (i.e., C limitation) rather than soil acidification caused changes in aggregate-associated microbial groups and subsequent reductions in microbial biomass in bulk soil. Plants allocate less C to belowground parts when N/P availability is relatively rich [1,17]. This could directly reduce bacterial uptake for C in SMA and MA, resulting in their energy deficiency and then limiting bacterial growth in these aggregates. However, this was not the case for the bacterial response in LMA. Relative to bacteria, fungi rely much on root-derived C [4,16] that primarily accumulated in LMA [46]. The view was supported by the

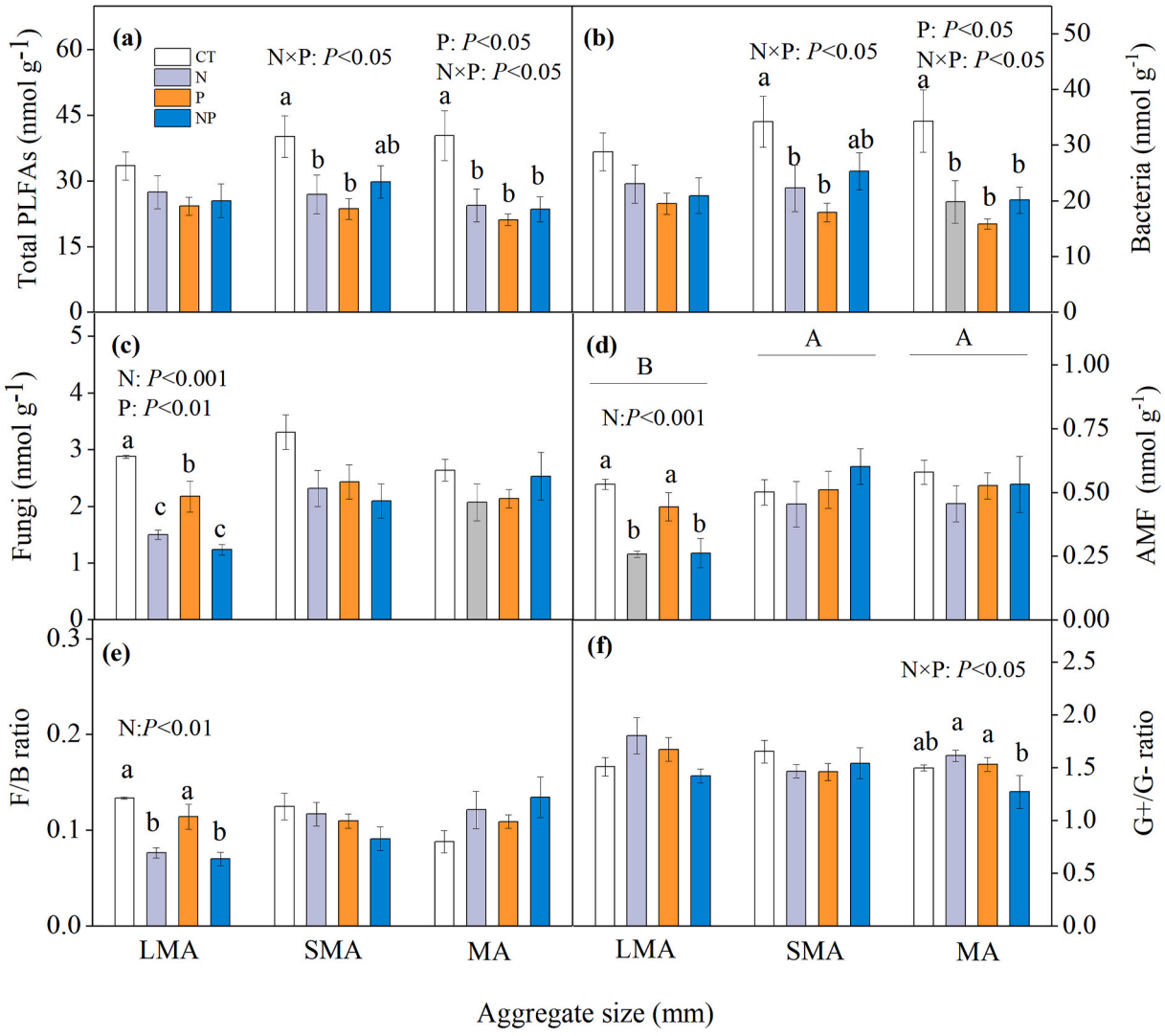

Fig. 2. Effects of N fertilization, P fertilization, and N plus P fertilization on microbial communities across different aggregate sizes. Error bars indicate mean ± SE (n = 3). Different lowercase letters indicate significant differences among nutrient treatments within the same aggregate fraction. Different uppercase letters represent significant differences among aggregate fractions. Aggregates were classified into three fractions: LMA (>2 mm), SMA (0.25–2 mm) and MA (<0.25 mm).

stronger correlations between fungi in LMA and root biomass in the present study (Fig. 5). Thus, N-induced decline in root input led to lower fungi in LMA (Fig. 2c). This could alleviate fungal antagonistic effects towards bacterial growth [35] and provide their residues for utilization by bacteria [13], which may counteract the negative root effects on bacteria in LMA after N fertilization and make the bacterial response in LMA to N fertilization non-significant.

Subtropical forests with highly weathered soils have long been considered P-deficient [48]. Therefore, P inputs often stimulates microbial growth in these forests [18,21]. Nonetheless, contrary to this common belief and our initial hypothesis, we clearly found that single P fertilization significantly suppressed total PLFAs (Fig. 1a), suggesting P is not a limiting factor for microbial biomass in the studied plantation. Since P effect on aggregate-associated microbial groups was similar to N-only fertilization (Fig. 2a, b, and 2c), the above-mentioned mechanism also works to explain P effects on microbial biomass. The different responses of soil microbial biomass to P fertilization among studies may be attributed to their divergent soil P status and harvest practices. For example, the concentration of soil available P was three times higher in the current study than in a secondary tropical forest [18]. Moreover, understory and litter biomass were continuously kept in our study, unlike an earlier study in a tropical forest in which they were removed until the late 1990s [20]. Nevertheless, the unchanged AMF under P fertilization (Fig. 2d) is rather unexpected, because negative P effect on AMF abundance has been consistently reported across global experiments (reviewed in Ma et al. [25]). The driving mechanism of this finding awaits further investigation. Possibly, P-induced decline in root biomass alleviates the competition between plants and AMF for other nutrients, which promotes the dominance of certain AMF species and consequently, offsets the negative effect of P fertilization. Another possible explanation is that estimation of AMF biomass using PLFA 16:1w5 might mask P effect on the real AMF since there is a strong background by bacterial-derived PLFA 16:1w5 [5]. Therefore, future efforts will be needed to uncover the effect of P fertilization on AMF with alternative methods for accurately estimating their biomass.

Despite the strong P effect, microbial biomass did not differ among N, P and NP treatments (Fig. 1a), contrary to our first hypothesis. This result is mainly due to the following reasons. First, globally, simultaneous N and P fertilization stimulates above-ground biomass more than when they are added separately [19]. Such greater promotions in above-ground productivity could partially offset the stronger microbial C limitation caused by lower root inputs under NP fertilization than single N or P fertilization. Second, N-induced soil acidification (Table 1) promotes the accumulation of toxic ions, such as Al^{3+} [3], which poisons

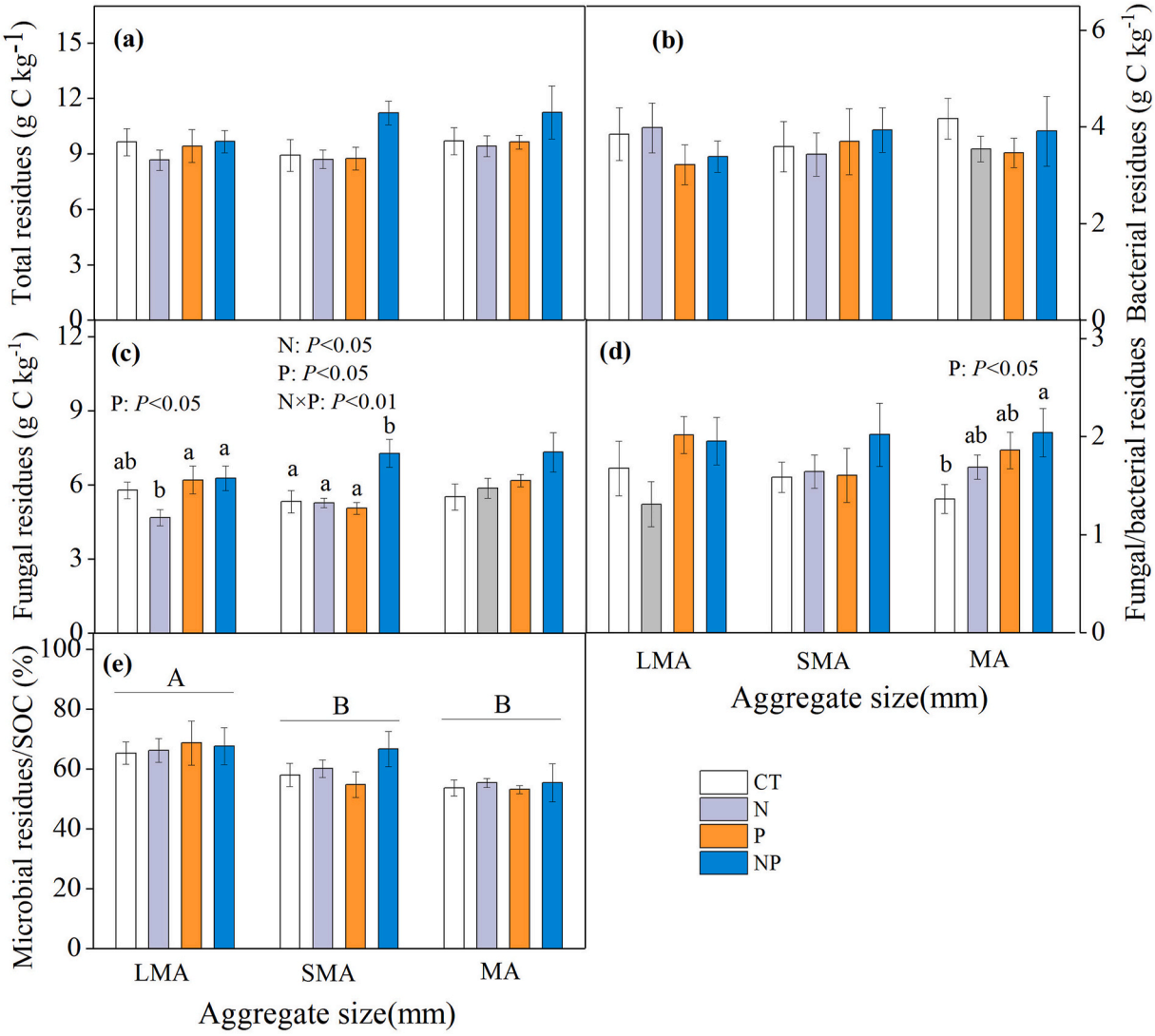

Fig. 3. Effects of N fertilization, P fertilization, and N plus P fertilization on microbial residues across different aggregate factions. Error bars indicate mean ± SE (n = 3). Different lowercase letters indicate significant differences among nutrient treatments within the same aggregate fraction. Different uppercase letters represent significant differences among aggregate fractions. Aggregates were classified into three fractions: LMA (>2 mm), SMA (0.25–2 mm) and MA (<0.25 mm).

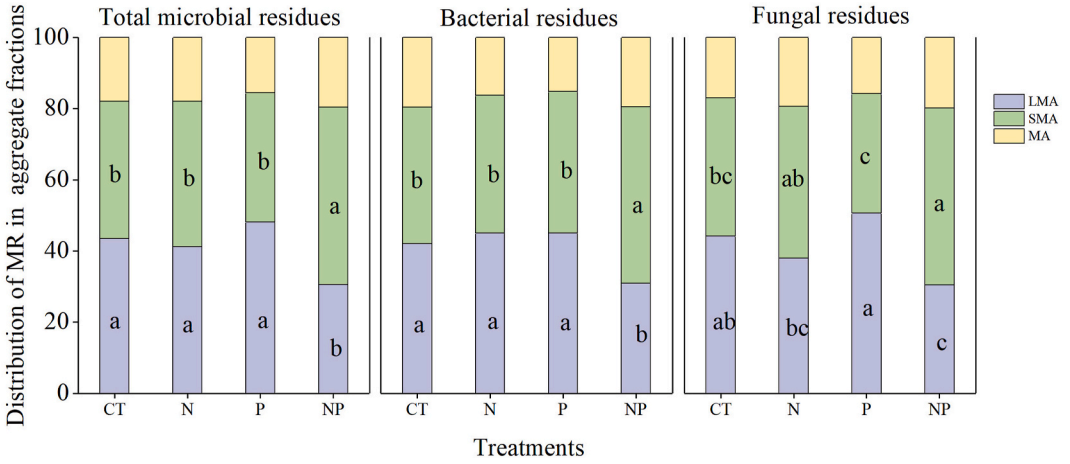

Fig. 4. Effects of N fertilization, P fertilization, and N plus P fertilization on the distribution of total (a), bacterial (b) and fungal residues across aggregate fractions to bulk soil. Different lowercase letters indicate significant differences among nutrient treatments within the same aggregate fraction at $P < 0.05$. MR, microbial residues. Aggregates were classified into three fractions: LMA (>2 mm), SMA (0.25–2 mm) and MA (<0.25 mm).

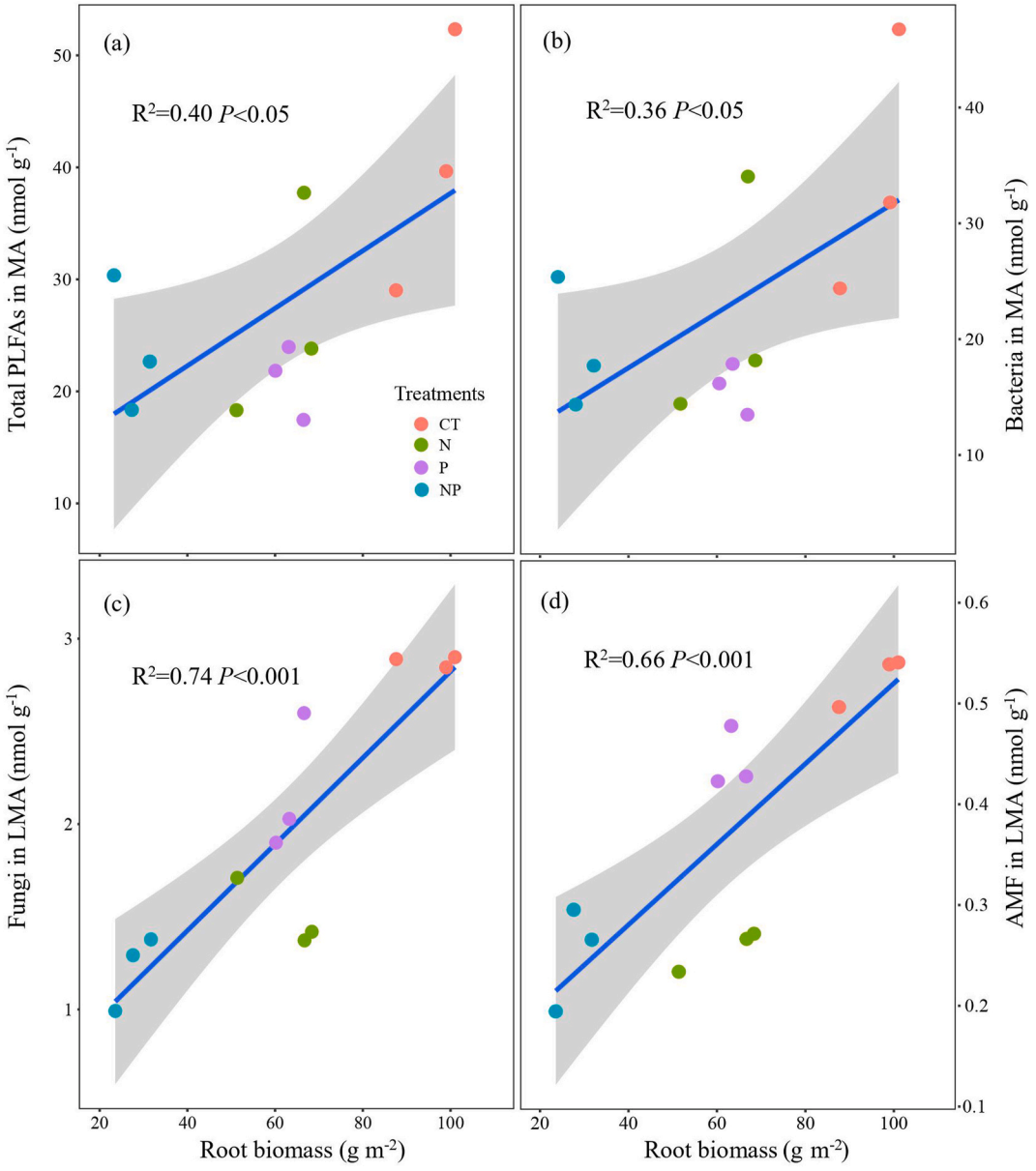

Fig. 5. Relationships of aggregate-associated microbial groups with root biomass under fertilization. The solid blue line represents the fitted regression line and the grey shading represents 95% confidence interval. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

microbial cells. The added P can combine with Al^{3+} when pH < 6.5 and alleviate aluminum poisoning [47]. These together could explain the non-additive interaction of N and P fertilization on microbial biomass.

4.2. *Effects of fertilization on microbial residues*

In contrast to the observed changes in microbial biomass, microbial residues and their contribution to SOC in bulk soils remained stable under different fertilization treatments (Fig. S2). This result indicates that microbial residue accumulation differs from that of living microorganisms, because it integrates changes in microbial communities over time and its linear relationship with microbial biomass can be altered by its self-degradation [20]. Similarly, previous studies found that 6–11 years of fertilization (mainly N fertilization) do not affect microbial residue accumulation across different forests [23,24]. In contrast, a study conducted in a tropical *Castanopsis carlesii* forest found that the contribution of microbial residues to SOC increased after 7-year N addition [24]. One explanation for such differences is that the decrease magnitude of SOC to N fertilization was much smaller in our study than in other work (4% in this study vs. 9% in a previous study).

Interestingly, when soils were categorized into different aggregate fractions, simultaneous N and P fertilization significantly stimulated fungal residue accumulations in SMA, although adding them separately did not (Fig. 3c). These findings indicate that N inputs can significantly modify the effect of P fertilization on fungal residue accumulations in aggregates. Contrary to our study, a previous study [49] found that the negative effect of P fertilization on aggregate-associated fungal residues was independent on N fertilization in a tropical coastal forest. Such inconsistent results indicate that the response of fungal residues at aggregate scale to NP fertilization is highly context-dependent. SEM showed that NP fertilization directly and indirectly stimulated the proportion of SMA via root-driven aggregate breakdown (Fig. 6). This results in fungal residues to be preferentially preserved in soils over bacterial residues because large fungal fragments can be easily occluded

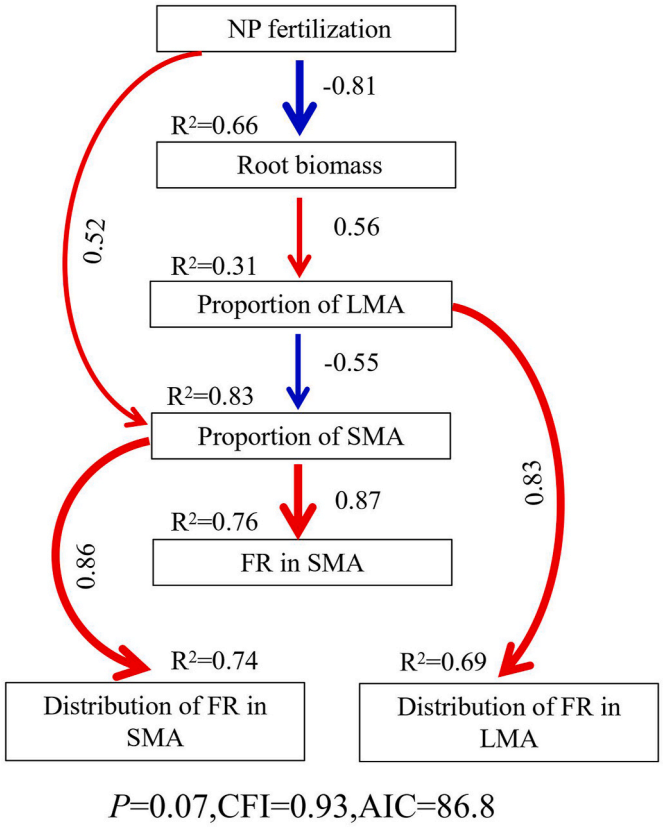

Fig. 6. Structural equation model (SEM) for controls of aggregate-associated fungal residues and their distribution. Results of model fitting: $P = 0.07$, CFI = 0.93, AIC = 86.8. Red lines represent positive paths, blue lines represent negative paths, and the width of lines indicates the effect size. The values along the lines indicate the effect size. FR, fungal residues; LMA, > 2 mm aggregate; SMA, 0.25–2 mm aggregate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inside SMA (Fig. 6) [8] and containing refractory substances such as melanins makes fungal residues more resistant to biodegradation [12]. On the other hand, greater loss of root biomass under NP fertilization than under single N/P fertilization (Table 1) broke LMA to SMA (Fig. 6) because roots generally enmesh small aggregates into large ones [32, 38]. This further releases protected highly bioavailable C fractions in LMA [29], provides an energy and C source for microbial growth, thereby alleviating the use of fungal residues in SMA. Notably, the changes in soil aggregate structure under NP fertilization further shifted microbial and fungal residues from LMA to SMA (Figs. 4 and 6). Given that smaller aggregate fractions have stronger physical protection of SOC [38], the present finding suggests that NP fertilization appeared to increase C stabilization via increasing microbial residue distribution in SMA. Taken together, our results suggest that predicting soil C dynamic based on single fertilization experiments and ignoring physical protection of microbial residue-C may be misleading. Therefore, future studies that explore the response of the soil C pool to N and/or P fertilization should focus on microbial residues at the aggregate scale in subtropical plantation ecosystems.

5. Conclusions

Understanding the interactive effects of multiple fertilization on soil microbes at finer soil scales is important for accurately predicting soil C sequestration. By isolating soil aggregate fractions from bulk soil, this study reveals that N and/or P fertilization decreased bacteria in SMA and MA and fungi in LMA, which jointly resulted in a significant inhibition in total microbial biomass in bulk soils. Fertilization affected the microbial biomass mainly through decreased root biomass rather than soil acidification. By contrast, NP fertilization increased fungal residues in SMA and further redistributed microbial residues from LMA to SMA, mainly through stimulating the proportion of SMA. Thus, for Chinese fir plantation, NP fertilization seems to be an effective practice to maintain long-term fertility of plantation soil via increasing the stability of soil microbial residues. Overall, these results suggest that results from single fertilization manipulation experiments and ignoring physical protection of microbial residue-C may have limited power to predict soil C dynamics in the future. This work offers new insights into the interactions of N and P fertilization and provides an important guidance for management practices in subtropical conifer plantations.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2021.103376.

References

[1] K. Bae, T.J. Fahey, R.D. Yanai, M. Fisk, Soil nitrogen availability affects belowground carbon allocation and soil respiration in northern hardwood forests of New Hampshire, Ecosystems 18 (2015) 1179–1191.
[2] R.D. Bardgett, P.J. Hobbs, A. Frostegard, Changes in soil fungal:Bacterial biomass ratios following reductions in the intensity of management of an upland grassland, Biol. Fertil. Soils 22 (1996) 261–264.
[3] W.D. Bowman, C.C. Cleveland, L. Halada, J. HreskoJ, S. Baron, Negative impact of nitrogen deposition on soil buffering capacity, Nat. Geosci. 1 (2008) 767–770.
[4] J.B. Brant, D.D. MyroldE, W. Sulzman, Root controls on soil microbial community structure in forest soils, Oecologia 148 (2006) 650–659.
[5] T. Camenzind, J. Homeier, K. Dietrich, S. Hempel, D. Hertel, A. Krohn, C. Leuschner, Y. Oelmann, P.A. Olsson, J.P. Suarez, M.C. Rilli, Opposing effects of nitrogen versus phosphorus additions on mycorrhizal fungal abundance along an elevational gradient in tropical montane forests, Soil Biol. Biochem. 94 (2016) 37–47.
[6] Z. Chen, X. Zhou, S. Geng, Y. Miao, S.J. Han, Interactive effect of nitrogen addition and throughfall reduction decreases soil aggregate stability through reducing biological binding agents, For. Ecol. Manag. 445 (2019) 13–19.
[7] E.A. Davidson, R.W. Howarth, Environmental science: nutrients in synergy, Nature 450 (2007), 363-363.
[8] X. Ding, C. Liang, B. Zhang, Y. YuanX. Han, Higher rates of manure application lead to greater accumulation of both fungal and bacterial residues in macroaggregates of a clay soil, Soil Biol. Biochem. 84 (2015) 137–146.
[9] W.Y. Dong, X.Y. Zhang, X.Y. Liu, X.L. Fu, J.B. Chen, Responses of soil microbial communities and enzyme activities to nitrogen and phosphorus additions in Chinese fir plantations of subtropical China, Biogeosciences 12 (2015) 5537–5546.
[10] B. Engelking, H. Flessa, R.G. Joergensen, Shifts in amino sugar and ergosterol contents after addition of sucrose and cellulose to soil, Soil Biol. Biochem. 39 (2007) 2111–2118.
[11] H. Fang, J. Geng, S. Cheng, M. Xu, Z. Cao, Effects of nitrogen and phosphorus enrichment on carbon sequestration in forest soils: a review, Acta Pedol. Sin. 56 (2019) 1–11 (in Chinese).
[12] C.W. Fernandez, K. Heckman, R. KolkaP, G. Kennedy, Melanin mitigates the accelerated decay of mycorrhizal necromass with peatland warming, Ecol. Lett. 22 (2019) 498–505.
[13] J. Huang, W. Liu, M. Deng, X. Wang, Z. Wang, L. Yang, L. Liu, Allocation and turnover of rhizodeposited carbon in different soil microbial groups, Soil Biol. Biochem. 150 (2020) 107973.

[14] J.D. Jastrow, T.W. BouttonR, M. Miller, Carbon dynamics of aggregate-associated organic matter estimated by carbon-13 natural abundance, Soil Sci. Soc. Am. J. 60 (1996) 801–807.
[15] J. Jiang, Y.P. Wang, Y. Yang, M. Yu, C. Wang, J. Yan, Interactive effects of nitrogen and phosphorus additions on plant growth vary with ecosystem type, Plant Soil 440 (2019) 523–537.
[16] Y. Jing, Y. Wang, S. Liu, X. Zhang, Q. Wang, K. Liu, Y. Yin, J. Deng, Interactive effects of soil warming, throughfall reduction, and root exclusion on soil microbial community and residues in warm-temperate oak forests, Appl. Soil Ecol. 142 (2019) 52–58.
[17] N.C. Johnson, Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales, New Phytol. 185 (2010) 631–647.
[18] J. Li, Z. Li, F. Wang, B. Zou, Y. Chen, J. Zhao, Q. Mo, Y. Li, X. Li, H. Xia, Effects of nitrogen and phosphorus addition on soil microbial community in a secondary tropical forest of China, Biol. Fertil. Soils 51 (2015) 207–215.
[19] Y. Li, S. Niu, G. Yu, Aggravated phosphorus limitation on biomass production under increasing nitrogen loading: a meta-analysis, Global Change Biol. 22 (2016) 934–943.
[20] C. Liang, W. Amelung, J.M.K. Lehmann, Quantitative assessment of microbial necromass contribution to soil organic matter, Global Change Biol. 25 (2019) 3578–3590.
[21] L. Liu, P. Gundersen, W. Zhang, T. Zhang, H. Chen, J. Mo, Effects of nitrogen and phosphorus additions on soil microbial biomass and community structure in two reforested tropical forests, Sci. Rep. 5 (2015) 14378.
[22] R. Lu, Methods of Soil Agricultural Chemistry Analysis, Chinese Agricultural Science and Technology Press, Beijing, 2000, pp. 24–26.
[23] S. Ma, G. Chen, E. Du, D. Tian, A. Xing, H. Shen, C. Ji, C. Zheng, J. Zhu, J. Zhu, H. Huang, H. He, B. Zhu, J. Fang, Effects of nitrogen addition on microbial residues and their contribution to soil organic carbon in China's forests from tropical to boreal zone, Environ. Pollut. 268 (2021) 115941.
[24] S. Ma, G. Chen, D. Tian, E. Du, W. Xiao, L. Jiang, Z. Zhou, J. Zhu, H. He, B. Zhu, J. Fang, Effects of seven-year nitrogen and phosphorus additions on soil microbial community structures and residues in a tropical forest in hainan island, China, Geoderma 361 (2020) 114034.
[25] X.C. Ma, Q.H. Geng, H.G. Zhang, C.Y. Bian, H.Y.H. Chen, D.L. Jiang, X. Xu, Global Negative Effects of Nutrient Enrichment on Arbuscular Mycorrhizal Fungi Plant Diversity and Ecosystem Multi-Functionality, New Phytol, 2020. https://doiorg/101111/nph17077.
[26] Z. Ma, X. Zhang, C. Zhang, H. Wang, F. Chen, X. Fu, X. Fang, X. Sun, Q. Lei, Accumulation of residual soil microbial carbon in Chinese fir plantation soils after nitrogen and phosphorus additions, J. Forensic Res. 29 (2018) 953–962.
[27] K.G. MacDicken, Global forest resources assessment 2015: what, why and how? Forest Ecol.Manag. 352 (2015) 3–8.
[28] A. Miltner, P. Bombach, B. Schmidt-Bruecken, M. Kaestner, SOM genesis: microbial biomass as a significant source, Biogeochemistry 111 (2012) 41–55.
[29] R. Murugan, I. Djukic, K. Keiblinger, F. Zehetner, M. Bierbaumer, S. Zechmeister-Bolternstern, R.G. Joergernsen, Spatial distribution of microbial biomass and residues across soil aggregate fractions at different elevations in the central austrian alps, Geoderma 341 (2019), 249-249.
[30] X. Ni, S. Liao, S. Tan, Y. Peng, D. Wang, K. Yue, F. Wu, Y. Yang, The vertical distribution and control of microbial necromass carbon in forest soils, Global Ecol. Biogeogr. 29 (2020) 1829–1839.
[31] J. Penuelas, B. Poulter, J. Sardans, P. Ciais, M. van der Velde, L. Bopp, O. Boucher, Y. Godderis, P. Hinsinger, J. Llusia, E. Nardin, S. Vicca, M. ObersteinerI, A. Janssens, Human-induced nitrogen-phosphorus imbalances alter natural and managed ecosystems across the globe, Nat. Commun. 4 (2013) 2934.
[32] V. Poirier, C. Roumet, D.A. Angers, A.D. Munson, Species and root traits impact macroaggregation in the rhizospheric soil of a mediterranean common garden experiment, Plant Soil 424 (2018) 289–302.
[33] K. Schermellehengel, H. Moosbrugger, H. Müller, Evaluating the fit of structural equation models: tests of significance and descriptive goodness-of-fit measures, Meth. Psychol. Re. 8 (2003) 23–74.
[34] J.P. Schimel, S.M. Schaeffer, Microbial control over carbon cycling in soil, Front. Microbiol. 3 (2012) 348.
[35] T. Schneider, T. Kruse, R. Wimmer, I. Wiedemann, V. Sass, U. Pag, A. Jansen, A. K. Nielsen, P.H. Mygind, D.S. Ravents, S. Neve, B. Ravn, A.M.J.J. Bonvin, L. De Maria, A.S. Andersen, L.K. Gammelgaard, H.G. Sahl, H.H. Kristensen, Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II, Science 328 (2010) 1168–1172.
[36] B. Seely, R. Welham, R.A.J.E.I. Blanco, Towards the application of soil organic matter as an indicator of forest ecosystem productivity: deriving thresholds, developing monitoring systems, and evaluating practices, Ecol. Indicat. 10 (2010) 999–1008.
[37] J. Six, P. Callewaert, S. Lenders, S. De Gryze, S.J. Morris, E.G. Gregorich, E.A. Paul, K. Paustian, Measuring and understanding carbon storage in afforested soils by physical fractionation, Soil Sci. Soc. Am. J. 66 (2002) 1981–1987.
[38] J. Six, E.T. Elliott, K.J.S. Paustian, Soil structure and soil organic matter: Ii. A normalized stability index and the effect of mineralogy, Soil Sci. Soc. Am. J. 64 (2000) 1042–1049.
[39] J. Six, H. Bossuyt, S. Degryze, K. Denef, A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics, Soil Till. Re. 79 (2004) 7–31.
[40] M. Stemmer, M.H. GerzabekE. Kandeler, Organic matter and enzyme activity in particle-size fractions of soils obtained after low-energy sonication, Soil Biol. Biochem. 30 (1998) 9–17.
[41] C. Tarnocai, J.G. Canadell, E.A.G. Schuur, P. Kuhry, G. Mazhitova, S. Zimov, Soil organic carbon pools in the northern circumpolar permafrost region, Global Biogeochem. Cycles 23 (2009) GB2023.
[42] J.M. Tisdall, J.M.J. Oades, Organic matter and water-stable aggregates in soils, J. Soil Sci. 62 (1982) 141–163.
[43] K.K. Treseder, Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies, Ecol. Lett. 11 (2008) 1111–1120.
[44] S.K. Tripathi, C.P. KushwahaK, K.P. Singh, Tropical forest and savanna ecosystems show differential impact of N and P additions on soil organic matter and aggregate structure, Global Change Biol. 14 (2008) 2572–2581.
[45] R. Wang, H. Wu, J. Sardans, T. Li, H. Liu, J. Penuelas, F.A. Dijkstra, Y. Jiang, Carbon storage and plant-soil linkages among soil aggregates as affected by nitrogen enrichment and mowing management in a meadow grassland, Plant Soil 457 (2020) 407–420.
[46] X. Wang, L. Yin, F.A. Dijkstra, J. Lu, P. Wang, W. Cheng, Rhizosphere priming is tightly associated with root-driven aggregate turnover, Soil Biol. Biochem. 149 (2020) 107964.
[47] Z. Wang, S. Yang, R. Wang, Z. Xu, K. Feng, X. Feng, T. Li, H. Liu, R. Ma, H. Li, Y. Jiang, Compositional and functional responses of soil microbial communities to long-term nitrogen and phosphorus addition in a calcareous grassland, Pedobiologia 78 (2020) 150612.
[48] G. Yu, Y. Jia, N. He, J. Zhu, Z. Chen, Q. Wang, S. Piao, X. Liu, H. He, X. Guo, Z. Wen, P. Li, G. Ding, K. Goulding, Stabilization of atmospheric nitrogen deposition in China over the past decade, Nat. Geosci. 12 (2019) 424–429.
[49] Y. Yuan, Y. Li, Z. Mou, L. Kuang, W. Wu, J. Zhang, F. Wang, D. Hui, J. Penuelas, J. Sardans, H. Lambers, J. Wang, Y. Kuang, Z.A. Li, Z. Liu, Phosphorus addition decreases microbial residual contribution to soil organic carbon pool in a tropical coastal forest, Global Change Biol. 27 (2021) 454–466.
[50] X.D. Zhang, W. Amelung, Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils, Soil Biol. Biochem. 28 (1996) 1201–1206.
[51] C. Zhang, X.Y. Zhang, Y. Kuzyakov, H.M. Wang, X.L. Fu, Y. Yang, F.S. Chen, J.A. J. Dungait, S.M. Green, X.M. Fang, Responses of C-, N- and P-acquiring hydrolases to P and N fertilizers in a subtropical Chinese fir plantation depend on soil depth, Appl. Soil Ecol. 150 (2020) 103465.
[52] C. Zhou, M. Wang, L. Zheng, Y Luo Jiang, Patterns and mechanisms of responses by soil microbial communities to nitrogen addition, Soil Biol. Biochem. 115 (2017) 433–441.