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Effects of simulated nitrogen deposition on soil microbial biomass and community function in subtropical evergreen broad-leaved forest

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Abstract

Aim of the study: The aim of this study was to examine the effects of a 5-year simulated nitrogen (N) deposition on soil microbial biomass carbon (MBC), nitrogen (MBN), microbial community activity and diversity in subtropical old-growth forest ecosystems.

Area of study: The study was conducted in forest located at subtropical forest in Anhui, east China.

Material and methods: Three blocks with three fully randomized plots of 20 m × 20 m with similar forest community and soil conditions were established. The site applied ammonium nitrate (NH₄NO₃) to simulate N deposition (50 and 100 kg N ha⁻¹ year⁻¹). From three depths (0–10, 10–20 and 20–30 cm), were collected over four seasons (December, March, June and September), and then measured by community-level physiological profiles (CLPPs).

Main results: N addition had no significant effect on MBC and MBN. The spatiotemporal variations in MBC and MBN were controlled by seasonality and soil depth. Soil microbial activities and diversity in the growing season (June and September) were apparently higher than the dormant season (March and December), there were significantly lower diversity indices found following N addition in September. However, N addition enhanced microbial activities and increased diversity indices in the dormant season. Redundancy analysis showed that pH, soil moisture, NO₃⁻-N and total phosphorus were the most important factors controlling the spatial pattern of microbial metabolic activity.

Research highlights: These results suggest that soil microbial community function is more easily influenced than microbial biomass. The site has a trend of P-limited or near-N saturation, and will threaten the whole forest ecosystem with the increasing duration of N addition.

Additional keywords: Nitrogen deposition; Seasonality; Soil microbial biomass; Microbial community; Subtropical old-growth forest.

Authors' contributions: Conceived and designed the experiments: Xu, X. and Wang, J.; Performed the experiments: Teng, Z., Guan, M. and Zhao, X.; Analyzed the data: Wang, J., Cui, J. and Fan, W.; Drafting of the manuscript: Wang, J.; Critical revision of the manuscript for important intellectual content: Xu, X.

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Introduction

Due to the burning of fossil fuels, the production and use of chemical fertilizer and other interferences caused by human activity, anthropogenic reactive nitrogen (N) is increasing and is deposited by precipitation and through dust accumulation in terrestrial and aquatic ecosystems

(Zhang *et al.*, 2015). Worldwide N deposition has caused serious environmental problems, such as the acidification of soil and water, the imbalance of soil–nutrient C/N ratios, increased leaching of soil nitrate and nutrients, and loss of biodiversity (Bobbink *et al.*, 2010; Stevens *et al.*, 2018). However, N deposition has the potential to constrain the accumulation of

anthropogenic CO₂ in the atmosphere by increasing ecosystem carbon (C) storage. This could thereby slow the pace of climate warming (Xia & Wan, 2008; Frey *et al.*, 2014), for which forest ecosystems play an important role by sequestering atmospheric CO₂. In addition, soils can contribute to C sequestration. Soil C sequestration and storage in forested ecosystems are mediated by soil microbial communities and are in dynamic equilibrium with environmental factors (Lal, 2005; Zhu *et al.*, 2017).

Soil microorganisms are essential regulators of nutrient turnover in forest ecosystems (Wardle *et al.*, 2004). They are involved in key soil biogeochemical processes including mineralization and humification of organic matter in soils (Cotrufo *et al.*, 2013; Xia *et al.*, 2016). The turnover rate of soil organic matter may depend on the microbial community structure and the capacity of the community to metabolize different organic compounds (Garcia-Pausas & Paterson, 2011). N addition is predicted to increase microbial biomass via increasing C or/and N resource availabilities; thus, increased N deposition could alter the structure and composition of soil microbial communities because of different competition among species of soil microorganisms for various forms of N (Schimel & Bennett, 2004; Van Diepen *et al.*, 2010; Zechmeister-Boltenstern *et al.*, 2011). N deposition can affect microorganisms in different ways in different ecosystems. Previous studies have shown that high N deposition significantly reduced soil microbial biomass and decreased rhizodeposition, fueling rhizosphere communities (Mo *et al.*, 2010; Treseder, 2010; Eisenhauer *et al.*, 2012). However, to date, the results are inconsistent. Some studies found that high N addition had no effect on soil microbial characteristics (Keeler *et al.*, 2009). These inconsistent results indicated that the response of soil microbial communities to N deposition is highly variable among different environments. Therefore, more detailed studies should be conducted on this topic.

The application of a community level method to determine microbial function provides a more sensitive and ecologically sensitive method for determining heterotrophic microbial community structure. This method employs the commercial Biolog microtiter plate

assay (Biolog Inc., Hayward, CA) for monitoring shifts in the metabolic patterns of microbial communities (Garland, 1997; Campbell *et al.*, 2003). In recent years, the method has been widely used in the studies of soil microbial communities, which mainly included evaluations of the effects of different forest types, N deposition, and various factors (such as drought, fertilizers, contaminants, changes in plant cover) on catabolic abilities of soil microorganisms (Bérarda *et al.*, 2011; Banning *et al.*, 2012; Brackin *et al.*, 2013; Sradnick *et al.*, 2013).

In this study, we designed a long-term (>4 years) N addition experiment in an old-growth subtropical evergreen broad-leaved forest, which faces high atmospheric N deposition (over 10 kg N ha⁻¹ yr⁻¹), to test the following two hypotheses: (1) N addition has negative effects on microbial biomass and functional capacity of soil microorganisms; and (2) Seasonal variations are much greater than the effects of N addition on the functional ability of the soil microbial community. These changes may be due to changes in species dominance, or they may be due to the constant physiological adaptation of soil microorganisms. The functional capacity of the soil microbial community was also analyzed in relation to chemical and physical soil variables.

Materials and methods

Site description, experimental design and sampling

The N-addition experimental site was established in an old-growth subtropical evergreen broad-leaved forest at Mt. Xianyu in Shitai County, Anhui Province, eastern China (30°01'47" N, 117°21'23" E, 375 m a.s.l.). The study area belongs to the humid subtropical monsoon climate, with a mean annual temperature and mean annual precipitation of 16°C and 1626 mm, respectively. The stand structure of the forests and soil chemical properties of the study site are given in Table 1. The forest was dominated by evergreen broad-leaved tree species *Castanopsis eyrei* and *Cyclobalanopsis glauca*, with a diverse subcanopy of tree species.

Table 1. Stand structure of the sampling forests and soil chemical properties.

Soil type	Stand feature				Property of the surface soil layer (0–10 cm)				
	Density (tree ha ⁻¹)	Basal area (m ² ha ⁻¹)	Mean DBH (cm)	pH (H ₂ O)	Organic C (g kg ⁻¹)	Total N	Total P	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N
Yellow brown	1316±157	33.5±4.7	14.2±1.6	4.48±0.15	53.7±6.8	2.23±0.37	0.27±0.05	11.23±2.13	1.27±0.51

DBH: diameter at breast height of tree; Values are mean ±standard deviation (SD).

The forests at our site have been well-protected from management disturbances over the past 50 years. The background N deposition rates were estimated at 10.7 kg N ha⁻¹ yr⁻¹ (Zhao *et al.*, 2013). We applied ammonium nitrate (NH₄NO₃) at the level of 50 (N50) or 100 kg N ha⁻¹ year⁻¹ (N100) to simulate N deposition. Control plots (CK) received no N addition. The NH₄NO₃ was dissolved in 20 L of stream water and evenly sprayed on the soil surface of the sampling plots every two months, using a backpack sprayer from June 2011 (Du *et al.*, 2013). The same amount of stream water (20 L) was applied to control plots in each treatment event. Three blocks with three fully randomized plots of 20 m × 20 m with similar forest community and soil conditions were established; there are 10 m isolates between each plot to prevent interference between different treatments (Zhao *et al.*, 2013).

The soil samples, from three depths (0–10, 10–20 and 20–30 cm), were collected over four seasons (December 2015, March, June and September 2016). Each plot was partitioned into five subplots along a diagonal direction; three soil cores were taken within the subplots and the same depths in the same plots were mixed in situ to form one pooled sample per depth per plot (removal of litter, roots and gravel). The soil samples were transported in cooled boxes to the laboratory immediately after sampling, sieved (2 mm mesh) and stored at 4°C under aerobic conditions for physicochemical and microbial activity measurements.

Soil physicochemical properties

The measured soil physicochemical properties included soil moisture (approximately 20 g subsample was oven-dried at 105°C for 24 h), pH (water suspension with a 1:2.5, soil:water ratio), NH₄⁺-N and NO₃⁻-N (fresh soil samples were extracted using a solution of 2 M KCl, measured by FIA Star 5000, FOSS, Denmark), soil organic carbon, total nitrogen (air-dried soil, measured by EA 3000, Vector, Italy), and total phosphorus (air-dried soil, H₂SO₄-HClO₄ digestion, measured by FIA Star 5000) (Zhao *et al.*, 2013).

Soil microbial analysis

Soil microbial biomass C (MBC) and N (MBN) were measured by a chloroform fumigation-extraction method. Fumigated and nonfumigated soil samples were extracted with 0.5 M K₂SO₄. Total N and dissolved organic carbon (DOC) in the extracts were immediately determined using a TOC automatic analyzer (Multi C/N 3100, Jena Analytik, Germany), and the difference in concentrations gave the amounts of

soil MBC and MBN, with conversion factors of 0.45 and 0.54, respectively (Vance *et al.*, 1987).

Microbial metabolic activity was determined using a 96-well Biolog EcoPlate™ (Biolog Inc., Hayward, California, USA). Ten grams of fresh soil were suspended in 100 ml of 0.85% sterile NaCl solution, shaken for 30 min on a reciprocal shaker, and diluted 1000-fold. Aliquots (150 µl) of the diluted sample were inoculated directly into Eco-microplates and incubated at 25°C in the dark without shaking. Due to the asymptotic nature of color development, the rate of postculture using average well color development (AWCD) values will be less accurate, so we cultured the microorganisms to 168 h (Vance *et al.*, 1987). Plates were read every 24 h at 590 nm for 168 h using a Biolog MicroStation™ (BIO-TEK Instruments Inc., Winooski, USA). The AWCD value was calculated for each sample at each time point by using the following equation (Gomez *et al.*, 2006):

$$AWCD = \sum OD_i / 31 \quad (1)$$

where OD_i is the optical density value from each well. Microbial functional diversity was assessed calculating the Shannon diversity index and the Simpson index, which were calculated using the following equations (Nair & Ngouajio, 2012; Zhu *et al.*, 2017):

$$\begin{aligned} \text{Shannon } (H') &= -\sum P_i \ln P_i \text{ and} \\ \text{Simpson } (D') &= 1 - \sum P_i^2, \end{aligned} \quad (2)$$

where P_i is the ratio of the corrected absorbance value of each well to the sum of absorbance values of all wells.

Statistical analysis

Considering our experimental design and our focus on the differences of microbial biomass and community function in different seasons with or without N fertilization, we used linear mixed-effect models to test the effects of N fertilization (fixed effect), seasons (fixed effect) and soil depths (fixed effect) on microbial biomass, soil nutrients and pH. In the model, the three blocks were used as a random-effects variable. Residual tests were performed using the Shapiro Test. Furthermore, to compare microbial biomass, soil nutrient, pH and microbial diversity across N treatments and seasons in each block, we used one-way ANOVA. When the results of one-way ANOVA showed a significant difference, we used the Tukey's honest significant difference (HSD) test to conduct multi comparisons among three N treatments and four seasons at each soil depth, respectively.

Ward cluster analysis and principal component analysis (PCA) were based on standardized Euclidean distance using the microbial community AWCD and diversity indices data at 72 h (Kumar *et al.*, 2016). The redundancy analysis (RDA) was used to visualize the shifts of microbial community characteristics between the N treatments and seasons. To explore which environmental variables explained most of the variation in microbial community, we included soil moisture, pH, soil organic C (SOC), total N (TN), total phosphorus (TP), NH_4^+ -N and NO_3^- -N as explanatory variables. All statistical analyses were performed with the statistical software R (version 3.5.0), using the packages of nlme, multcomp, vegan, ade4, gelus, cluster and ggplot2, etc. (Oksanen *et al.*, 2012).

Results

Soil nutrient content and pH

One-year monitoring of soil nutrient dynamics showed that N addition increased the contents of SOC, TN, NH_4^+ -N and NO_3^- -N significant, but decreased TP and pH (Fig. 1, Table 2; Fig. S1 [suppl.]). SWC showed a significant downward trend in September, while NO_3^- -N was significantly higher in September than other months. The significant seasonal variation of TP was that September and June were obvious higher than December and March. The effect of N addition on soil nutrients mitigated with decreasing soil depth (Figs. S1-S3 [suppl.]).

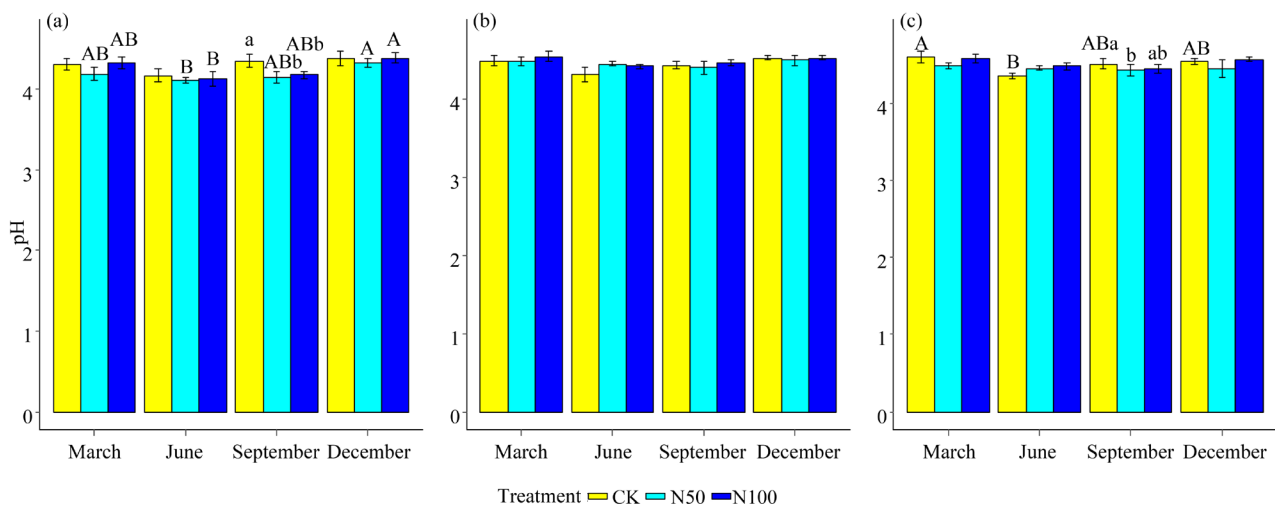


Figure 1. Changes in soil pH in different N treatments. (a) 0–10 cm, (b) 10–20 cm, (c) 20–30 cm. Different lowercase letters indicate significant differences between N treatments during the same season; different capital letters indicate significant differences between seasons in the same N treatment. Bars represent mean \pm standard deviation (SD).

Soil microbial biomass

Seasonal variation and soil depth had significant effects on MBC, MBN and MBC/MBN, which decreased with soil depth (Fig. 2, Figs. S4-S5 [suppl.], Table 2). However, N addition showed no significant effect in most seasons (Fig. 2). The overall trend of change of MBC was December > September > March > June. The MBN was also significantly higher in December than in other months. MBC and MBN in June and September had a positive response to N addition, while in March, MBN showed a negative response to N addition. Overall, N addition increased the MBC/MBN ratio (Fig. 2).

Soil microbial activity and diversity

Seasonal variation had a significant effect on soil microbial activity and diversity indices, and microbial activity decreased with soil depth (Figs. 3 and 4, Figs. S6-S8 [suppl.]). In June and September, microbial activity and the Shannon diversity index were higher than December and March. N addition showed negative effects on microbial activity in June and September; however, N addition promoted microbial activity in December and March (Fig. 3). Simpson diversity and Shannon diversity indices were opposite in seasonal change. N addition decreased the microbial diversity in September, while increasing it in December (Fig. 4, Figs. S7-S8 [suppl.]). The soil microbial diversity index and activity were clustered into two groups. In March and December, they were concentrated on the negative axis of PCA1, and in June and September, they were

Table 2. Effects of predictors (i.e., N treatment, season, soil depth and their interactions) on soil microbial biomass, nutrient content and pH values tested with linear mixed-effects models.

Predictor	Df	SWC	pH (H ₂ O)	Organic carbon	Total N	Total P	HN ₄ ⁺ -N	NO ₃ ⁻ -N	MBC	MBN	MBC:MBN
				(g kg ⁻¹)	(mg kg ⁻¹)	(mg kg ⁻¹)	(mg kg ⁻¹)				
Treat	2	18.4***	2.5	21.9***	12.5***	32.6***	22.8***	29.3***	3.5*	4.5*	0.5
Season	3	81.8***	10.1***	1.7	3.6*	21.6***	5.5**	34.4***	300.9***	90.0***	96.9***
Depth	2	93.0***	56.9***	177.7***	157.5***	18.9***	59.1***	5.5*	14.5***	141.0***	23.8***
Treat: Depth	4	0.3	1.1	3.2*	2.7*	1.9	5.0**	0.4	0	1.2	0.7
Treat: Season	6	1.3	1.2	0.4	0.5	0.4	1.6	3.7**	3.0*	2.6*	1.2
Depth: Season	6	3.1**	0.7	1.2	2.4*	0.8	1.2	2.8*	5.3***	0.2	0.9

F-values with an asterisk indicate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Treat: N-addition treatments of CK, N50 and N100. Depth: soil depths of 0–10 cm, 10–20 cm and 20–30 cm. Season: spring (March), summer (June), autumn (September) and winter (December).

concentrated on the positive axis of PCA1. N addition had a slight influence on their spatial heterogeneity (Fig. S9 [suppl.]).

Substrate utilization efficiency by the microbial community

The 31 substrates in the Eco-microplate were classified into six categories (Phenolic compounds, Amines, Polymers, Carboxylic acids, Carbohydrates and Amino acids) (Garland & Mills, 1991). The different microbial communities had significantly different responses to carbon sources and seasonality. In March, amino acids, carboxylic acids and polymers were the dominant C sources for the microbial community. The utilization of amino acids was decreased, while

the utilization of carboxylic acids was increased by N addition. In June and September, N addition increased the microbial community using phenolic compounds. Amino acids, carbohydrates and carboxylic acids were the dominant C sources for the soil microbial community in December. N addition decreased the utilization of carbohydrates and carboxylic acids. In different soil layers, significant changes in utilization of phenolic compounds were observed in March and December (Fig. S10 [suppl.]).

Redundancy analysis of the soil microbial community

Under the constraints of environmental factors, the effect of seasonal variation on soil microbial community

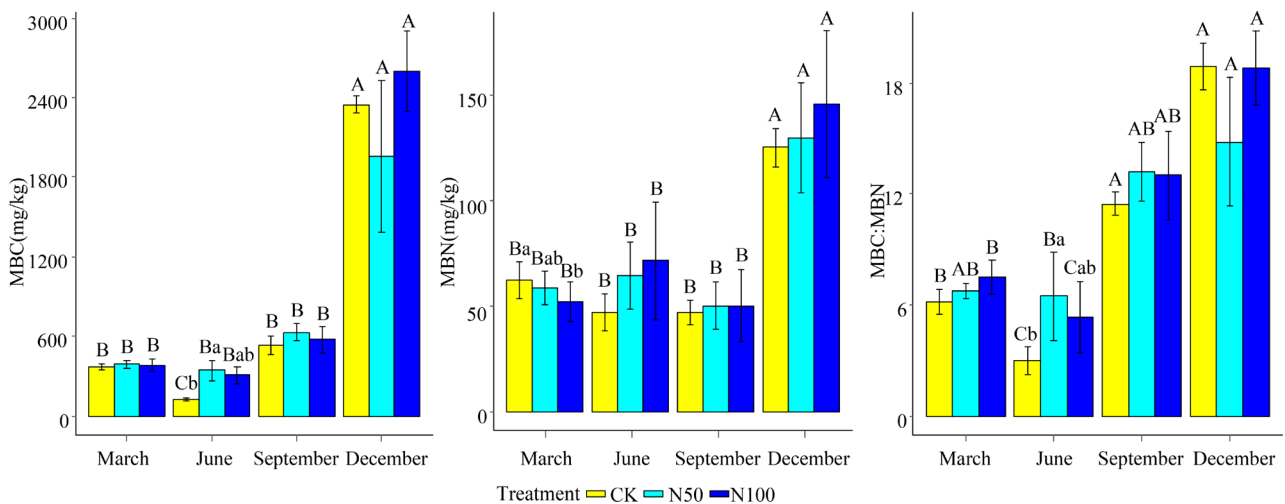


Figure 2. Changes of soil MBC, MBN and MBC/MBN in different N treatments at the surface soil layer (0–10 cm). Different lowercase letters indicate significant differences between N treatments during the same season; different capital letters indicate significant differences between seasons in the same N treatment ($p < 0.05$). Bars represent mean \pm standard deviation (SD).

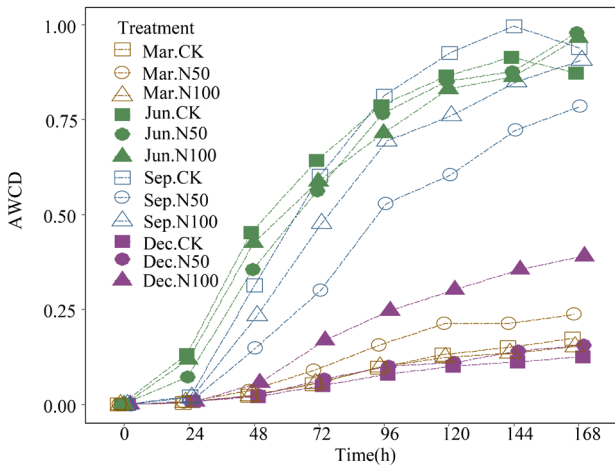


Figure 3. Changes of the average well color development (AWCD) in different N treatments at the surface soil layer (0–10 cm).

was still obvious. The contribution rate of RDA1 and RDA2 in the 0–10 cm soil layer was 74.44%. SWC, NO₃⁻-N and TP were the most important environmental factors affecting the metabolic capacity of the microbial community. The spatial heterogeneity of the metabolic capacity of the soil microbial community with different N availabilities was larger in September and was mainly affected by soil NO₃⁻-N and TP (Fig. 5). The contribution rates of RDA1 and RDA2 in the 10–20 cm and 20–30 cm soil layers were 86.13% and 77.79%, respectively. Among, pH, SWC and NO₃⁻-N were the most important factors controlling the functional distribution of the soil microbial community (Figs. S11-S12 [suppl.]).

Discussion

The soil microbial biomass comprises only approximately 2-3% of soil organic carbon, but it is an important factor regulating ecosystem processes. Our results indicate that soil microbial biomass in subtropical forest ecosystems exhibited a significant seasonal pattern, increasing in autumn and winter and decreasing in spring and summer. The similar seasonality was observed in other ecosystems such as alpine (Lipson *et al.*, 2000), warm temperate (Zhao *et al.*, 2011) and subtropical (Feng *et al.*, 2009). Seasonal variation in soil microbial biomass is controlled by environmental factors (e.g., soil moisture, temperature, pH, nutrient and C availability), plant physiological factors and organic matter input (Wardle, 1998; Feng *et al.*, 2009; Kaschuk *et al.*, 2010). In the subtropical region, the resource availability in soil is usually high due to the rapid turnover of organic matter. However, during the growing season, strong competition may occur between vegetation and soil microbial communities. This may result in low microbial biomass caused by resource limitation for the microbial communities. A decreasing temperature in winter would weaken plant physiological activities and enhance organic matter input by litter and fine-root turnover. This results in a significant change of soil microbes between winter and summer (Lipson *et al.*, 2000). Conflicting reports on seasonal changes in soil microbial biomass exist (Wardle, 1998; Nieder *et al.*, 2010). Further research is required to reveal the mechanisms regulating the seasonal patterns of soil microbial biomass in different forest ecosystems.

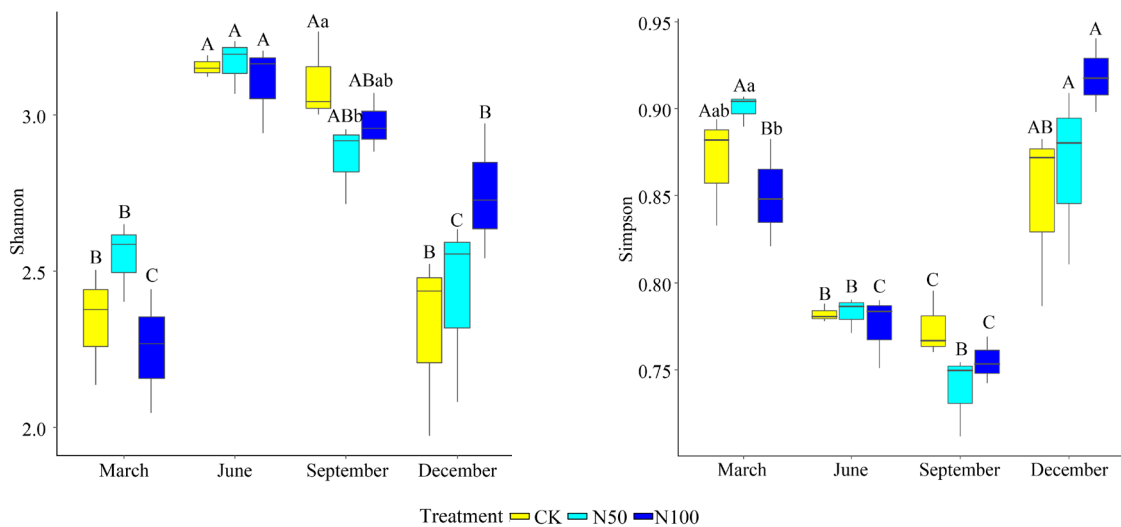


Figure 4. Changes of soil microbial community diversity in different N treatments at the surface soil layer (0–10 cm). Different lowercase letters indicate significant differences between N treatments during the same season; different capital letters indicate significant differences between seasons in the same N treatment ($p < 0.05$). Boxplots are divided by 5%, 25%, median, 75% and 95% of the data.

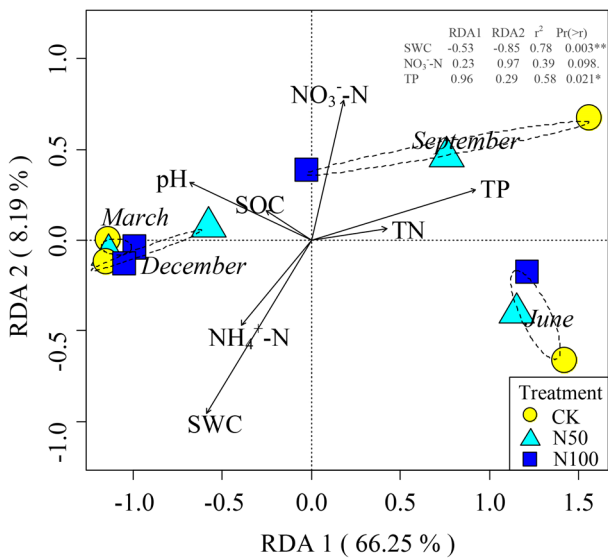


Figure 5. Redundancy analysis of the utilization of 31 carbon sources by the soil microbial community at the surface soil layer (0–10 cm). Each ordination point uses the average of three blocks. Asterisks indicate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Due to the low content of N and P in the acidic soil in this region (Table 1), the addition of N can alleviate the N limitation of the forest ecosystem (Di *et al.*, 2017). The addition of N increased the TN content of the soil; meanwhile, the TP content was reduced, and thus, the soil N/P ratio increased. Therefore, N addition is likely to drive P-limitation in this subtropical evergreen broad-leaved forest. In forest ecosystems, soil microorganisms are the key factors determining biodiversity, productivity and energy turnover (Heijden *et al.*, 2010). A recent Meta-analysis based on 151 experiments showed that atmospheric N deposition negatively affects soil microbial growth, composition, and function across all terrestrial ecosystems, with more pronounced effects with increasing N deposition rate and duration (Zhang *et al.*, 2018). Nevertheless, other studies found that the effects of N addition on soil microbial biomass and community structure differ greatly among different forest types (Keeler *et al.*, 2009; Allison *et al.*, 2010; Mo *et al.*, 2010). For nutrient addition in subtropical forests in particular, there is still great uncertainty (Balser *et al.*, 2001). In this study, there were no significant differences in soil microbial biomass, community activity and diversity between N-amended and control plots. The mechanism is that food and energy required by soil microorganisms are mainly limited by soil carbon. N addition may lead to changes in soil carbon availability, or the unbalanced distribution of soil carbon from above- and below-ground, which may have indirect adverse effects on the survival and reproduction of soil microorganisms.

Generally, the soil nutrient availability, enzyme activity and the change of community structure in the aboveground part can affect the soil microbial community to a great extent. Jing *et al.* (2017) reported that soil extracellular enzyme activity had no obvious response to N addition at our site. Cui (Cui *et al.*, 2017) found that N addition had no significant effect on soil microbial diversity in this area by 16S analysis. These results, which indicate that soil microbial survival does not respond to N addition is indicative of N saturation at our site, which would require a long-term N addition experiment to verify.

The results of the RDA showed that the soil microbial community remained relatively stable under the N addition in this study. It is generally believed that soil moisture and pH are two key factors affecting soil microbial community and function (Bobbink *et al.*, 2010; Brockett *et al.*, 2012). At our site, differences in soil microbial communities caused by N addition and seasonal factors are largely explained by pH, SWC, NO₃⁻-N and TP (Fig. 5, Fig. S11-12 [suppl.], $p < 0.05$). Among various soil properties that affect the soil microbial community structure, pH is a key factor. This is in agreement with many studies, especially in those conducted in acidic soil of warm and humid areas (Leff *et al.*, 2015; Zhang *et al.*, 2017; Zhang *et al.*, 2018). When soil pH is lower than 4.5, soil microorganisms will be significantly inhibited (Högberg *et al.*, 2007; Johannes *et al.*, 2009). After 5 years of N addition, soil pH showed a small decline. The change of soil pH in this region had no significant effect on microbial diversity (Cui *et al.*, 2017). In addition, root exudates and litterfall production in forest ecosystems are controlled by vegetation development and hydrothermal conditions and are the main carbon sources for microbial communities (Priha *et al.*, 2001). Di *et al.* (2017) showed that the understory vegetation (such as saplings, surface ferns and herbaceous plants) were sensitive to N addition. This should contribute to the changes of the soil microbial community and its activity.

Conclusion

Our five-year experiment showed that simulated N deposition had no significant effects on soil microbial biomass, community metabolic activity or functional diversity in a subtropical old-growth forest ecosystem. The main soil factors resulting in the change of soil microbial community were pH, soil moisture, NO₃⁻-N and total P. This indicates that N addition may likely drive P limitation in this subtropical forest. In addition, the physiological changes of understory vegetation

and aboveground litterfall production affected by N addition cannot be ignored and could be an important contributor to the changes of soil microbial community and its activity. Due to the global and long-term nature of atmospheric N deposition, its impact on forest ecosystems is bound to be long-term and to entail complicated processes.

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