# Effects of Nitrogen and Phosphorus Addition on Soil Microbial Community Characteristics in Subtropical Evergreen Broad-Leaved Forests: Postprint

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## Abstract

To investigate the effects of nitrogen and phosphorus addition on soil microbial characteristics, we established a nitrogen and phosphorus addition experiment with four treatments in an evergreen broad-leaved old-growth forest in Xianyu Mountain, Chizhou, Anhui Province: control (CK, 0 kg N/hm$^2$), low nitrogen (LN, 50 kg N/hm$^2$), high nitrogen (HN, 100 kg N/hm$^2$), and high nitrogen plus phosphorus (HN+P, 100 kg N/hm$^2$ + 50 kg P/hm$^2$). Using the chloroform fumigation method and Biolog microplate technique, we analyzed the effects of different nitrogen and phosphorus addition levels on soil microbial biomass carbon (MBC), nitrogen (MBN), and microbial community functional diversity across different soil depths (0-10 cm, 10-20 cm, and 20-30 cm). The results demonstrated that MBC and MBN decreased with soil depth, with highly significant differences, and both MBC and the MBC/MBN ratio exhibited significant differences following nitrogen and phosphorus addition. The metabolic activity of soil microbial communities also decreased with soil depth, with the highest activities observed under HN and LN treatments. The McIntosh, Shannon, and Simpson diversity indices varied across soil layers and nitrogen/phosphorus addition levels, with more pronounced differences observed in surface soils. Soil microorganisms exhibited the highest utilization rates for carboxylic acids, amino acids, and carbohydrate carbon sources. Principal component analysis revealed distinct patterns in carbon source utilization by soil microorganisms across different soil layers, with significant spatial variability in surface soil microbial carbon source utilization under different nitrogen and phosphorus addition levels, while other layers showed more concentrated distributions. Spatial differences were primarily manifested in the utilization of carbohydrate and carboxylic acid carbon sources. Both soil depth and nitrogen/phosphorus addition rates significantly influenced soil microbial biomass carbon, nitrogen, and functional diversity, with high nitrogen treatment exerting the greatest impact on surface soil microorganisms.

## Full Text

## Preamble

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**Effects of Nitrogen and Phosphorus Addition on Soil Microbial Community Characteristics in a Subtropical Evergreen Broad-Leaved Forest**

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## Abstract

To investigate the effects of nitrogen and phosphorus addition on soil microbial characteristics, we selected an old-growth evergreen broad-leaved forest in the Xianyu Mountains of Chizhou, Anhui Province. Four nitrogen and phosphorus addition treatments were established: control (CK, 0 kg N/ha), low nitrogen (LN, 50 kg N/ha), high nitrogen (HN, 100 kg N/ha), and high nitrogen plus phosphorus (HN+P, 100 kg N/ha + 50 kg P/ha). Soil microbial biomass carbon (MBC), biomass nitrogen (MBN), and microbial community functional diversity were analyzed for different soil layers (0–10 cm, 10–20 cm, and 20–30 cm) using chloroform fumigation and Biolog microplate techniques. The results showed that MBC and MBN decreased significantly with soil depth. Both MBC and the MBC/MBN ratio changed significantly after N and P addition. The metabolic activity of the soil microbial community also declined with soil depth, with the highest activities observed in HN and LN treatments. The McIntosh, Shannon, and Simpson diversity indices differed across soil layers and N/P addition levels, with the most significant differences found in the surface soil layer (0–10 cm). Soil microorganisms exhibited highest utilization rates for carboxylic acids, amino acids, and carbohydrates. Principal component analysis revealed distinct patterns in carbon source utilization among soil layers, with significant spatial variability in surface soil microbial carbon source utilization across different addition treatments, while deeper layers showed more concentrated distributions. This spatial heterogeneity was primarily manifested in the utilization of carbohydrates and carboxylic acids. In conclusion, both soil layer and N/P addition treatments significantly impacted MBC, MBN, and soil microbial functional diversity, with the greatest effects occurring in the surface soil under high N treatment.

**Keywords:** nitrogen and phosphorus addition; evergreen broad-leaved forest; soil microbial community; functional diversity; microbial biomass C and N

## 1. Introduction

Soil microorganisms are the most active components of soil and drive soil nutrient cycling. The flow of materials and energy cycling in soil are accomplished through the coordination of different microorganisms, with carbon and nitrogen cycles being central to these processes [1]. Soil microorganisms continuously decompose organic matter, regulating the transformation of organic compounds and nutrient release. Microbial biomass serves as both a source and sink of soil nutrients and is a sensitive indicator of soil fertility [2]. Long-term fertilization practices not only affect soil physicochemical properties but also alter conditions such as moisture, pH, osmotic pressure, and temperature required for microbial growth and development, thereby changing soil microbial characteristics [3]. Soil microbial community functional diversity reflects the ecological characteristics of soil microbial communities and has become a hot topic in ecological research [4].

Nitrogen and phosphorus are essential macronutrients for plant growth and play important roles in plant development and reproduction. Many ecosystems exhibit nitrogen-limited productivity, with nitrogen fixation by diazotrophs being the primary pathway for nitrogen entry into terrestrial ecosystems under natural conditions. Phosphorus enters ecosystems mainly through the weathering of primary minerals. Ecosystems in early successional stages typically show nitrogen limitation, while those in later stages are primarily phosphorus-limited, with some systems showing co-limitation [5]. Reactions controlling nitrogen availability are mainly biological, whereas those controlling phosphorus availability are geochemical [6]. Nitrogen is also an active nutrient element in soil, existing in both organic and inorganic forms. Nitrogen transformations include ammonification, nitrification, denitrification, and biological immobilization. Nitrification and ammonification are processes that increase nitrogen availability for plant uptake.

Human activities have greatly increased nitrogen inputs through inorganic fertilizer application to promote plant growth. However, excess nitrogen use can significantly impact forest ecosystem structure and function, and soil microbial responses to nitrogen addition are diverse [7]. Therefore, rational nitrogen and phosphorus addition is crucial for sustainable forest ecosystem management. Subtropical evergreen broad-leaved forests are widely distributed in China, with rich biodiversity, complex community structure, high biomass and productivity, and good ecological benefits. They play important roles in water conservation, soil retention, and biodiversity maintenance [8]. While some studies have reported on nitrogen addition effects on soil nitrogen cycling in evergreen broad-leaved forests, the effects of phosphorus addition on soil microorganisms across different soil layers remain poorly understood.

This study established experimental plots in the Xianyu Mountain scenic area of western Huangshan and conducted five consecutive years of nitrogen and phosphorus addition experiments to investigate changes in soil microbial biomass carbon and nitrogen under different fertilization gradients. Biolog microplate analysis was used to examine the effects of different fertilization treatments on soil microbial community functional diversity, providing a scientific basis for carbon and nitrogen transformation monitoring, soil fertility assessment, and development of fertilization models to improve forest productivity.

## 2. Study Area and Methods

### 2.1 Study Area Description

The experimental site was located in the Xianyu Mountain scenic area in Shitai County, Anhui Province (30°01 47.44 N, 117°21 23.33 E) at an elevation of 375 m. The region has a subtropical humid climate with an average annual temperature of 16°C and annual precipitation of 1620 mm. The soil type is yellow-brown earth with acidic reaction. Vegetation is rich, with extensive areas of Chinese fir (*Cunninghamia lanceolata*), deciduous broad-leaved forest, and masson pine (*Pinus massoniana*). Zonal forest vegetation below 600 m is primarily subtropical evergreen broad-leaved forest, while evergreen-deciduous broad-leaved mixed forest occurs above 600 m. According to local records, the experimental forest is a protected "fengshui forest" with no human management for many years. The experimental stand is dominated by *Castanopsis eyrei* with high tree species richness. Based on baseline surveys, the experimental plots had similar stand structure, with 36 tree and shrub species (DBH $\geq$ 5.0 cm). Main associated tree species include *Cyclobalanopsis glauca*, *Cinnamomum subavenium*, *Nyssa sinensis*, *Dendropanax dentiger*, *Symplocos stellaris*, and *Litsea coreana*. Main shrub species include *Symplocos sumuntia*, *Camellia cuspidata*, and *Elaeocarpus sylvestris*. Herbaceous species such as *Tricyrtis macropoda* and *Ophiorrhiza japonica* are also present, with high overall species richness.

### 2.2 Experimental Design

The experiment included four treatments: control (CK, 0 kg N/ha), low nitrogen (LN, 50 kg N/ha), high nitrogen (HN, 100 kg N/ha), and high nitrogen plus phosphorus (HN+P, 100 kg N/ha + 50 kg P/ha). Nitrogen fertilizer was applied as ammonium nitrate ($NH_4NO_3$) and phosphorus fertilizer as calcium superphosphate ($Ca(H_2PO_4)_2$). Each treatment had three replicates. Fertilizer was dissolved in water and applied monthly using a sprayer. Fertilization continued for five consecutive years.

### 2.3 Soil Sampling

Twelve fixed plots (20 m × 20 m each) were established with 8-10 m buffers between plots to prevent cross-treatment interference. Soil samples were collected in March, July, August, September, and November 2015 using an S-shaped sampling pattern. Soils were collected from three layers (0–10 cm, 10–20 cm, and 20–30 cm), mixed thoroughly, and divided into two portions. One portion was used for physicochemical analysis, while the other was sieved (2 mm) to remove litter, placed in sterile bags, and refrigerated at 4°C for microbial community functional diversity analysis.

### 2.4 Soil Physicochemical Properties and Microbial Biomass Measurement

Soil water content was determined by the oven-drying method. Soil pH was measured in a 2.5:1 water-to-soil mixture. Ammonium nitrogen and nitrate nitrogen were analyzed using a flow injection analyzer (FIAstar 5000, FOSS, Denmark). Total carbon and total nitrogen were determined using an elemental analyzer (EA 3000, Vector, Italy). Dissolved organic carbon was measured using a Multi C/N 3100 analyzer (Jena Analytik, Germany). Microbial biomass carbon (MBC) and nitrogen (MBN) were measured using the chloroform fumigation method. Baseline soil properties are shown in .

### 2.5 Soil Microbial Community Functional Diversity Measurement

Fresh soil samples were placed in 250 mL Erlenmeyer flasks with 100 mL of 0.85% NaCl solution, shaken at 170 rpm for 30 minutes, and then diluted. The diluted solution was refrigerated for 30 minutes, after which the supernatant was poured into sterile petri dishes. Using an eight-channel pipette, 150 L of the solution was added to each well of Biolog-ECO microplates. Plates were incubated in a biochemical incubator, with data read automatically at regular intervals. The average well color development (AWCD) was used to indicate microbial community carbon source utilization capacity and overall metabolic activity [10].

### 2.6 Data Processing

AWCD was calculated as: $\mathrm{AWCD} = \Sigma(Ci - R)/31$, where Ci is the absorbance value of the ith well and R is the absorbance of the control well. If $Ci - R < 0$, the value was recorded as 0. Soil microbial functional diversity indices (McIntosh, Shannon, and Simpson) were calculated following standard formulas [11]. Statistical analyses including one-way ANOVA, two-way ANOVA, cluster analysis, and principal component analysis were performed using SPSS 17.0 software.

## 3. Results

### 3.1 Effects of Different Fertilization Treatments on Soil Physicochemical Properties

Long-term N and P addition significantly affected soil properties, particularly ammonium nitrogen and nitrate nitrogen, which showed extremely significant differences among treatments and soil layers. Two-way ANOVA results are presented in . Total nitrogen content in HN-treated soil was lower than CK in the 20–30 cm layer. Total phosphorus content varied significantly among treatments, with HN+P treatment showing higher values than other treatments in the 0–20 cm layer. Ammonium and nitrate nitrogen contents in the 0–20 cm layer were significantly lower in CK than in fertilized treatments. Available phosphorus in HN+P treatment was significantly higher than in other treatments, while nitrate nitrogen in the 10–30 cm layer was lower in HN+P than in LN and HN treatments. Detailed soil physicochemical properties under different treatments are shown in .

### 3.2 Effects of Different Treatments on Soil Microbial Biomass Carbon and Nitrogen

Soil microbial biomass carbon, nitrogen, and the MBC/MBN ratio showed highly significant differences among soil layers. MBC also differed significantly among fertilization treatments, while MBN differences were less pronounced. No significant interaction effects were observed between layer and treatment. Both MBC and MBN decreased with soil depth. In the 0–10 cm layer, MBC followed the pattern: CK > HN+P > LN > HN; in 10–20 cm: HN > CK > LN > HN+P; and in 20–30 cm: HN > CK > HN+P > LN. MBN patterns varied by layer as shown in and [Figure 1: see original paper].

### 3.3 Effects of Different Treatments on Soil Microbial Community Carbon Source Utilization Dynamics

AWCD values increased gradually with incubation time, entering logarithmic growth phase where microbial metabolic activity was most vigorous and carbon sources were heavily utilized, then slowing until plateauing. Surface soil (0–10 cm) showed higher overall absorbance values than deeper layers. In the 0–10 cm layer, AWCD followed: LN > HN > HN+P > CK; in 10–20 cm: HN > HN+P > LN > CK; and in 20–30 cm: HN > LN > HN+P > CK. Two-way ANOVA of AWCD values is presented in , and temporal changes are shown in [Figure 2: see original paper].

### 3.4 Effects of Different Treatments on Soil Microbial Community Diversity Indices

The McIntosh, Shannon, and Simpson diversity indices, which reflect species composition and distribution, showed significant differences among treatments and soil layers. Surface soil (0-10 cm) microbial diversity indices differed significantly among treatments, with HN and HN+P treatments showing distinct differences from others. Diversity indices for 10-30 cm layers also showed significant variation. Detailed diversity indices are presented in . Cluster analysis of diversity indices across soil layers and treatments is shown in [Figure 3: see original paper].

### 3.5 Effects of Different Treatments on Soil Microbial Community Carbon Source Utilization

Soil microorganisms showed highest utilization rates for carboxylic acids, amino acids, and carbohydrates. Carbon source utilization patterns were similar across soil layers. In the 0-10 cm layer, CK, LN, and HN treatments showed higher carbohydrate utilization than carboxylic acids, while HN+P showed the opposite pattern. In 10-20 cm and 20-30 cm layers, LN and HN treatments showed highest utilization of polymers and carboxylic acids. HN+P treatment showed preference for carboxylic acids across all layers. Overall, carbohydrate and amino acid utilization was highest, followed by polymers, with amines and polyphenols being lowest. Carbon source utilization efficiency across soil layers is shown in [Figure 4: see original paper].

### 3.6 Principal Component Analysis of Soil Microbial Community Carbon Source Utilization

Principal component analysis (PCA) of carbon source utilization across soil layers and treatments showed that PC1 and PC2 explained 76.47% and 6.65% of variance respectively (cumulative 83.12%) in the 0-10 cm layer; 52.25% and 7.71% (cumulative 65.87%) in the 10-20 cm layer; and 44.54% and 10.86% (cumulative 54.92%) in the 20-30 cm layer. PC1 was the main component driving variation in microbial community carbon source utilization. Surface soil showed clear spatial variability in carbon source utilization among treatments, while deeper layers were more concentrated. The main carbon sources contributing to separation were carbohydrates and carboxylic acids. PCA results are presented in [Figure 5: see original paper], and correlation coefficients between carbon sources and principal components are shown in .

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## 4. Discussion

Soil microbial biomass carbon and nitrogen are important indicators of soil fertility and nutrient cycling. This study demonstrated that MBC and MBN decreased with soil depth, with surface layers showing significantly higher values, consistent with soil organic matter distribution patterns. The MBC/MBN ratio can reflect microbial community structure information [16]. The higher MBC/MBN ratio under HN treatment suggests that changes in microbial community structure may be the main reason for higher microbial biomass.

Microbial metabolic activity decreased with soil depth, with surface soils showing greater carbon substrate utilization capacity. The ability of microorganisms to utilize different carbon sources depends largely on microbial types and their inherent properties. The Biolog method measures microbial utilization of single carbon sources to compare community-level diversity [17]. Diversity indices reflect differences in microbial species number and evenness. Significant differences in McIntosh, Shannon, and Simpson indices across treatments indicate that surface soil microbial communities differed in their utilization of common carbon sources and species evenness.

Cluster analysis showed that surface soil (0–10 cm) diversity indices grouped separately from deeper layers (10–30 cm), indicating that N and P addition significantly affected surface soil microbial communities. Studies have shown that litter and root exudates are the main carbon sources for soil microorganisms [19]. Natural broad-leaved forests have complex community structures with high root biomass and exudates, providing abundant carbon sources that support diverse and functionally active microbial communities [20]. Plant diversity affects soil microbial functional metabolism through differences in litter composition [21].

Soil microorganisms showed increased utilization of phenolic carbon sources with increasing nitrogen addition rates. Phenolic compounds affect the natural balance of soil microorganisms, and high N treatment may help reduce phenolic accumulation and inhibit harmful microbial propagation. Amino acids are important components of soil organic nitrogen, and microorganisms can use them as nitrogen sources to synthesize plant growth regulators [22]. The increased utilization of amino acid carbon sources under HN treatment suggests that high N addition promotes soil organic matter decomposition. The spatial distribution differences in PCA reflect variations in the types and extent of carbon source utilization by soil microorganisms [24]. The greater dispersion of surface soil samples indicates lower functional stability in surface microbial communities compared to deeper layers.

The main carbon sources contributing to functional diversity separation were carbohydrates and carboxylic acids. Surface soils in natural broad-leaved forests have abundant litter and high root activity, supporting high microbial biomass and activity. Soil microbial metabolic activity and diversity decrease with depth due to deteriorating habitat conditions [25, 26]. Changes in microbial biomass carbon, nitrogen, and functional diversity depend on both soil depth and fertilization rate [27]. Higher rates of N and P addition can improve soil physicochemical properties, increase plant biomass and litter production, enhance microbial metabolic activity, and significantly affect microbial functional and species diversity.

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*Note: Figure translations are in progress. See original paper for figures.*