



# Effects of nitrogen and phosphorus additions on soil microbial community structure and ecological processes in the farmland of Chinese Loess Plateau

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**Abstract:** Microorganisms regulate the responses of terrestrial ecosystems to anthropogenic nutrient inputs. The escalation of anthropogenic activities has resulted in a rise in the primary terrestrial constraining elements, namely nitrogen (N) and phosphorus (P). Nevertheless, the specific mechanisms governing the influence of soil microbial community structure and ecological processes in ecologically vulnerable and delicate semi-arid loess agroecosystems remain inadequately understood. Therefore, we explored the effects of different N and P additions on soil microbial community structure and its associated ecological processes in the farmland of Chinese Loess Plateau based on a 36-a long-term experiment. Nine fertilization treatments with complete interactions of high, medium, and low N and P gradients were set up. Soil physical and chemical properties, along with the microbial community structure were measured in this study. Additionally, relevant ecological processes such as microbial biomass, respiration, N mineralization, and enzyme activity were quantified. To elucidate the relationships between these variables, we examined correlation-mediated processes using statistical techniques, including redundancy analysis (RDA) and structural equation modeling (SEM). The results showed that the addition of N alone had a detrimental effect on soil microbial biomass, mineralized N accumulation, and  $\beta$ -1,4-glucosidase activity. Conversely, the addition of P exhibited an opposing effect, leading to positive influences on these soil parameters. The interactive addition of N and P significantly changed the microbial community structure, increasing microbial activity (microbial biomass and soil respiration), but decreasing the accumulation of mineralized N. Among them, N<sub>24</sub>P<sub>12</sub> treatment showed the greatest increase in the soil nutrient content and respiration. N<sub>12</sub>P<sub>12</sub> treatment increased the overall enzyme activity and total phospholipid fatty acid (PLFA) content by 70.93%. N and P nutrient contents of the soil dominate the microbial community structure and the corresponding changes in hydrolytic enzymes. Soil microbial biomass, respiration, and overall enzyme activity are driven by mineralized N. Our study provides a theoretical basis for exploring energy conversion processes of soil microbial community and environmental sustainability under long-term N and P additions in semi-arid loess areas.

**Keywords:** nitrogen and phosphorus additions; microbial community structure; farmland ecosystem; nitrogen mineralization; soil enzyme activity

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## 1 Introduction

Soil microorganisms are a major component of terrestrial ecosystems that play an important role in the regulation of organic matter decomposition and plant growth (Palansooriya et al., 2019). Microbes not only mediate the migration of nutrients in the soil but also respond to nutrient inputs through changes in their community structure and biomass. Studying the functions and responses of soil microbial community structure to changes in soil nutrients can help us predict ecosystem responses to natural and human-induced global environmental changes (Chen et al., 2019). Over the past decades, human activities have released large amounts of nitrogen (N) and phosphorus (P) into terrestrial ecosystems (Galloway et al., 2008; Wang et al., 2015). This anthropogenically induced enrichment of N and P has profoundly affected the function of soil microbial community structure and geochemical cycle (He et al., 2016).

Although N enrichment can increase N availability in soils and alleviate N limitation in ecosystems, it can also cause soil acidification and alter soil microbial community structure (Widdig et al., 2020; Siegenthaler et al., 2022), affecting carbon (C) and N cycles of soil ecosystems (Nannipieri et al., 2018). Studies have shown that long-term addition of N in farmlands (Liu et al., 2018), grasslands (Cruz et al., 2009), and forest ecosystems (He et al., 2021) causes changes in community structure of soil bacteria and fungi by altering physical-chemical properties of soils (pH, ammonium, and nitrate N) (Niu et al., 2021). And some scholars have suggested that changes in microbial community structure are related to soil chemical properties (Zhang et al., 2022). Zhang et al. (2019) demonstrated that in N-constrained semi-arid grasslands, addition of N in reasonable amounts could enhance microbial biomass and respiration capacity, however, this effect was inhibited when N saturation reached. Changes in microbial activity and community structure can lead to further changes in soil extracellular enzyme activity, which drives soil organic matter decomposition and nutrient cycling (Chen et al., 2019). Based on the resource allocation theory of enzyme production (Allison and Vitousek, 2005), we can find that N addition can inhibit the activity of N-cycle enzymes and enhance the activity of hydrolases involved in C and P cycles. However, effects of nutrient addition on soil enzyme activity cannot be explained only by the resource allocation theory as soil enzyme activity is closely related to factors, such as microbial biomass (Wang et al., 2020).

Most studies in recent years have focused on the responses of soil microorganisms to N addition in forest and grassland ecosystems, whereas few studies have been conducted to determine the structure and function of microbial community in farmland ecosystems under conditions of P addition and its interaction with N in ecologically fragile loess areas. Frequently, the inputs of N and P are not synchronized, and unbalanced N and P inputs lead to changes in bioavailable N and P ratios (N:P), resulting in changes in microbial community structure and function (microbial communities are homeostatic and their C:N:P ratios are kept within a small range) (Schleuss et al., 2019), which highlights the urgent need to study the effects of N and P additions on farmland ecosystems independently and the interaction with each other. P fertilization has no noticeable effects on microbial community composition and load in agricultural soils (Shi et al., 2012, 2013). It increases soil microbial biomass and fungal:bacterial (F:B) ratio in tropical forests (Li et al., 2015), significantly reduces the abundance of fungal species in alpine meadow soils, and alters fungal community composition (He et al., 2016). Thus, the effect of P addition on soil microbial communities varies depending on the climate and ecosystem. Interestingly, Liu et al. (2021) found that P addition had no effect on soil respiration in grasslands; however, it had an amplified impact on respiration when N was added, and the two jointly affected the overall pattern of soil enzymes through soil eutrophication pathway.

The Loess Plateau is an important dry-farming area in northwestern China. However, fragile ecological environment, arid climate, and uneven precipitation distribution have become the primary obstacles in agricultural development of this region. The Chinese government has implemented a series of policies aimed at addressing environmental issues, such as the conversion of natural grasslands and croplands into forest or afforested lands (Wu et al., 2019). Vegetation

restoration has significantly increased the surface soil microbial biomass in the loess arid region (determined by phospholipid fatty acid (PLFA)), maintaining F:B ratio (due to similar increases in bacterial and fungal communities) (Cai et al., 2022). Bacterial communities have demonstrated dominance among overall soil microbial populations, while their composition has proven to be more sensitive to soil pH variations when compared with fungal communities (Zeng et al., 2021). The preservation and restoration of land productivity are influenced not only by the direct and indirect involvement of soil microorganisms in material and energy flow processes but also by the intervention of nutrient inputs (Han et al., 2021). Reasonable application of fertilizer is important for achieving a high crop yield and efficiency (Kracmarova et al., 2020). To increase crop production and agricultural income, local farmers use large amounts of chemical fertilizers, especially N and P fertilizers, which far exceed the needs of crops. Irrational fertilizer application limits crop yield and leads to soil pollution and eutrophication of water bodies, causing damage to ecological environment (Krauss et al., 2020). Enhancing field management practices and optimizing soil nutrient levels and microbial communities pose significant challenges in the endeavor to enhance utilization of arable land (Han et al., 2021). Therefore, it is important to determine the effects of different N and P ratios on soil biological traits for the efficient use of agricultural soils in semi-arid areas.

In this study, we investigated the changes in soil microbial community structure and ecological processes mediated by N and P additions for 36 a, further exploring the driving mechanisms between changes in microbial community structure and ecological processes. We also elucidated the direct and indirect effects of nutrient addition on microbial community and ecological processes. Based on previous findings, we hypothesized that: (1) addition of N and P would induce alterations in the structure of soil microbial community, possibly attributable to changes in nutritional environment for soil microorganisms; and (2) N addition alone affects soil ecological processes, including soil respiration, N mineralization, and enzyme activity, while interactive addition with P alleviates or amplifies this effect.

## 2 Materials and methods

### 2.1 Study area and experimental design

The experimental soil in our study was obtained from the Changwu Ecological Experiment Station of Chinese Academy of Sciences (35°14'N, 107°41'E), located in the central and southern Loess Plateau, China. The experimental station was 1200 m a. s. l. with a flat terrain and had a warm temperate semi-humid continental climate, i.e., monsoon climate, rainy in summer and autumn, and dry in winter and spring, with an average annual precipitation of 580 mm and a frost-free period of 171 d. It was a typical dry-farming area. The soil in the area was black loam, and winter wheat was the main crop.

The long-term locational fertilization trial began in 1984 with eighteen treatments, three replications per treatment, in a randomized blocks design with plots arranged in three rows, each trial plot measuring 4 m×8 m and having a 1-m buffer zone. In this study, we selected nine treatments in which high, medium, and low interactions of N and P were used for sampling. Nine treatments were as follows: CK (control, no fertilizer); N<sub>12</sub> (N fertilizer at 90 kg/hm<sup>2</sup>); N<sub>24</sub> (N fertilizer at 180 kg/hm<sup>2</sup>); P<sub>12</sub> (P fertilizer at 90 kg/hm<sup>2</sup>); N<sub>12</sub>P<sub>12</sub> (N and P fertilizer at 90 and 90 kg/hm<sup>2</sup>, respectively); N<sub>24</sub>P<sub>12</sub> (N and P fertilizer at 180 and 90 kg/hm<sup>2</sup>, respectively); P<sub>24</sub> (P fertilizer at 180 kg/hm<sup>2</sup>); N<sub>12</sub>P<sub>24</sub> (N and P fertilizer at 90 and 180 kg/hm<sup>2</sup>, respectively); and N<sub>24</sub>P<sub>24</sub> (N and P fertilizer at 180 and 180 kg/hm<sup>2</sup>, respectively). N and P fertilizers used were urea (46.4% N) and superphosphate (46.0% P<sub>2</sub>O<sub>5</sub>), respectively.

### 2.2 Sample collection

Soil samples were collected in May 2021 using five-point sampling with nine different treatments and three replications of each treatment. After sample collection, they were sent back to the

laboratory and materials, and plant-fallen roots were removed. Subsequently, the samples were passed through a 2-mm sieve and air-dried.

### 2.3 Soil physical-chemical analysis

Soil clay was measured with a Mastersizer 3000 laser analyzer (Mastersizer 3000, Malvern Instruments Ltd., Malvern, UK), and the data output conformed to the Kaczynski soil texture classification standard. Soil moisture content (SMC) was determined using oven drying method. Soil organic matter (SOM) content was analyzed using potassium dichromate external heating method. Soil pH was measured using a PHS-3C acidity meter. Soil total carbon (TC) and soil organic carbon (SOC) contents were analyzed using an elemental analyzer (Vario TOC, Elementar, Hanau, Germany). Soil total phosphorus (TP) was determined using concentrated sulfuric acid-perchloric acid digestion molybdenum-antimony anticolorimetric method. Soil available phosphorus (AP) was determined using sodium bicarbonate leaching molybdenum antimony resistance colorimetric method. Soil organic phosphorus (SOP) was determined by high temperature burning method. Soil total nitrogen (TN) content was determined using a Kjeldahl method. Soil nitrate nitrogen ( $\text{NO}_3^-$ -N) and ammonium nitrogen ( $\text{NH}_4^+$ -N) were extracted with 0.5 mol/L  $\text{K}_2\text{SO}_4$  solution after leaching, then they were determined by a continuous flow analyzer (Autoanalyzer 3, Bran-Luebbe, Hamburger, Germany). For the specific steps of experiment, we referenced from Bao (2000).

### 2.4 Soil microbial community and enzymatic activity analysis

Soil microbial community structure (Bunemann et al., 2004; Grayston et al., 2004) was determined using dipotassium hydrogen phosphate-trichloromethane method. Each fatty acid component was identified using a gas chromatograph equipped with a musical instrument digital interface (MIDI) software. We chose to characterize bacteria with PLFAs using i14:0, i15:0, a15:0, 15:0, i16:0, 10Me16:0, i17:0, a17:0, cy17:0, 17:0, br18:0, 10Me17:0, 18:1 $\omega$ 7, 10Me18:0, and cy19:0; fungi with PLFAs 18:2 $\omega$ 6 and 18:2 $\omega$ 9 (Baath and Anderson, 2003), and actinomycetes with PLFAs 10Me17:1 $\omega$ 7c, 10Me18:0, and 10Me16:0 (Zelles, 1997), respectively. PLFAs i14:0, i15:0, a15:0, i16:0, i17:0, and a17:0 were used as indicators of gram-positive bacteria (GP); and 16:1 $\omega$ 7c, cy17:0, 18:1 $\omega$ 7c, and cy19:0 were used as indicators of gram-negative bacteria (GN) (Fanin et al., 2019). Anaerobes were characterized using 14:0 DMA, 15:0 DMA, 16:0 DMA, i15:0 DMA, 16:1 $\omega$ 9c DAM, and cy19:0 DAM (Bossio et al., 1998).

Soil enzyme activities were analyzed using microplate fluorometric method (Saiya-Cork et al., 2002). Umbelliferone was used as a substrate for  $\beta$ -1,4-glucosidase (BG),  $\beta$ -N-acetylglucosaminidase (NAG), and alkaline phosphatase (AKP) activity. L-dihydroxyphenylalanine was used as a substrate for peroxidase (POD) and polyphenol oxidase (PPO) activity.

### 2.5 Soil microbial biomass, soil respiration, and mineralized nitrogen analysis

Soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were measured using chloroform fumigation-potassium sulfate extraction method (Saiya-Cork et al., 2002). Soil respiration was determined using lye absorption titration method (He et al., 2022). Twenty-five grams of soil sample was weighed and placed at the bottom of a 250-mL glass tissue culture bottle containing a small glass bottle with 5 mL of 0.5 mol/L NaOH. The flasks were incubated at 25°C for 7 d. After  $\text{CO}_2$  absorption by NaOH, the small beaker was removed, and 5 mL of 0.5 mol/L  $\text{BaCl}_2$  was immediately added to it. The beaker was sealed and then titrated with 0.125 mol/L hydrochloric acid. After continuous cultivation for 28 d, soil  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N contents before and after cultivation were measured by  $\text{K}_2\text{SO}_4$  leaching-flow analyzer, and soil nitrogen mineralization was calculated.

### 2.6 Statistical analysis

Soil respiration rate was determined by Equation 1:

$$R_t = \frac{(V_0 - V_t) \times \rho \times 44 \times 10^6}{2 \times 1000 \times t \times m}, \quad (1)$$

where  $R_t$  is the soil respiration rate (mg/(kg·d));  $t$  is the time (d);  $V_0$  is the amount of hydrochloric acid used in the titration blank (mL);  $V_t$  is the amount of hydrochloric acid used in the titration (mL);  $\rho$  is the hydrochloric acid concentration (mol/L); and  $m$  is the weighed soil mass (g). Respiratory entropy= $R_t$ /MBC.

The data are expressed as mean±standard deviation of three parallel sample determinations and using Origin 2017 to draw histograms. Statistical analysis was performed using SPSS v.25.0, and one-way analysis of variance (ANOVA) and least significant difference (LSD) methods were used for comparisons among soil samples with a significance level of 0.05. Redundancy analysis (RDA) was performed using CANOCO v.5.0. In addition, we used Amos Graphics software to construct structural equation models (SEM) to investigate the relationship between soil ecological processes following N and P additions.

### 3 Results

#### 3.1 Changes of soil physical-chemical properties

There was no significant difference in the composition of soil particle size among different treatments (Table 1). Compared with CK, high N addition treatments ( $N_{24}$ ,  $N_{24}P_{12}$ , and  $N_{24}P_{24}$ ) significantly reduced soil pH. Soil TP content in case of P addition treatments (P and NP) was significantly higher than those of other treatments, and showed a positive correlation with the concentration of P addition. The overall change in soil AP content was consistent with the change in TP. N addition significantly increased  $NO_3^-$ -N content of the soil ( $P < 0.05$ ), whereas P addition had no effect. Contents of TN,  $NH_4^+$ -N, SMC, and SOM in the soil of  $N_{24}P_{12}$  treatment increased by 16.47%, 55.17%, 10.43%, and 22.54%, respectively, compared with CK treatment, and were significantly higher than those of other treatments.

**Table 1** Soil physical-chemical properties under different nitrogen (N) and phosphorus (P) additions

Treatment	pH	TC (g/kg)	TN (g/kg)	TP (g/kg)	SOC (g/kg)	SOP (g/kg)	$NH_4^+$ -N (mg/kg)	$NO_3^-$ -N (mg/kg)	SMC (%)	AP (mg/kg)	SOM (g/kg)	Clay (%)	Physical clay (%)
CK	8.22± 0.03 <sup>a</sup>	17.49± 0.28 <sup>a</sup>	0.85± 0.02 <sup>c</sup>	0.73± 0.00 <sup>e</sup>	6.95± 0.14 <sup>d</sup>	0.08± 0.03 <sup>def</sup>	0.29± 0.04 <sup>bc</sup>	11.13± 0.08 <sup>e</sup>	19.95± 0.14 <sup>e</sup>	7.37± 0.46 <sup>e</sup>	11.98± 0.24 <sup>d</sup>	7.73± 0.37 <sup>a</sup>	36.95± 0.94 <sup>a</sup>
$N_{12}$	8.20± 0.06 <sup>a</sup>	16.85± 0.43 <sup>bc</sup>	0.72± 0.06 <sup>d</sup>	0.67± 0.01 <sup>f</sup>	6.33± 0.42 <sup>c</sup>	0.10± 0.01 <sup>cde</sup>	0.32± 0.04 <sup>abc</sup>	13.50± 0.29 <sup>c</sup>	16.78± 0.21 <sup>b</sup>	2.53± 0.15 <sup>f</sup>	10.91± 0.72 <sup>e</sup>	8.71± 1.43 <sup>a</sup>	34.19± 5.15 <sup>ab</sup>
$N_{24}$	7.92± 0.02 <sup>b</sup>	16.00± 0.49 <sup>c</sup>	0.90± 0.05 <sup>bc</sup>	0.69± 0.02 <sup>ef</sup>	7.70± 0.57 <sup>c</sup>	0.44± 0.03 <sup>a</sup>	0.23± 0.04 <sup>bc</sup>	25.11± 0.18 <sup>a</sup>	18.03± 0.04 <sup>s</sup>	4.30± 0.70 <sup>f</sup>	13.28± 0.98 <sup>c</sup>	7.65± 0.40 <sup>a</sup>	32.21± 2.61 <sup>ab</sup>
$P_{12}$	8.15± 0.09 <sup>a</sup>	17.04± 0.28 <sup>abc</sup>	0.85± 0.02 <sup>d</sup>	1.06± 0.01 <sup>c</sup>	7.10± 0.02 <sup>d</sup>	0.06± 0.02 <sup>f</sup>	0.22± 0.01 <sup>c</sup>	11.13± 0.11 <sup>e</sup>	18.97± 0.12 <sup>f</sup>	42.80± 0.69 <sup>c</sup>	12.24± 0.03 <sup>d</sup>	7.33± 0.71 <sup>a</sup>	32.59± 0.76 <sup>ab</sup>
$N_{12}P_{12}$	8.22± 0.05 <sup>a</sup>	16.96± 0.28 <sup>abc</sup>	0.75± 0.05 <sup>d</sup>	0.96± 0.04 <sup>d</sup>	7.92± 0.10 <sup>bc</sup>	0.13± 0.01 <sup>c</sup>	0.33± 0.10 <sup>abc</sup>	11.46± 0.12 <sup>d</sup>	20.59± 0.06 <sup>d</sup>	23.27± 1.89 <sup>d</sup>	13.66± 0.16 <sup>bc</sup>	7.93± 0.20 <sup>a</sup>	33.24± 2.19 <sup>ab</sup>
$N_{24}P_{12}$	7.88± 0.05 <sup>b</sup>	17.25± 0.04 <sup>ab</sup>	0.99± 0.06 <sup>a</sup>	0.94± 0.02 <sup>d</sup>	8.52± 0.09 <sup>a</sup>	0.17± 0.00 <sup>b</sup>	0.45± 0.02 <sup>a</sup>	14.54± 0.21 <sup>b</sup>	22.03± 0.04 <sup>a</sup>	25.00± 1.05 <sup>d</sup>	14.68± 0.15 <sup>a</sup>	7.55± 0.15 <sup>a</sup>	32.92± 2.96 <sup>ab</sup>
$P_{24}$	8.23± 0.05 <sup>a</sup>	16.17± 0.04 <sup>de</sup>	0.76± 0.01 <sup>d</sup>	1.32± 0.02 <sup>a</sup>	6.83± 0.11 <sup>de</sup>	0.07± 0.01 <sup>ef</sup>	0.38± 0.18 <sup>ab</sup>	10.88± 0.04 <sup>c</sup>	15.66± 0.07 <sup>i</sup>	66.77± 2.55 <sup>a</sup>	11.77± 0.19 <sup>de</sup>	7.30± 0.79 <sup>a</sup>	31.84± 2.48 <sup>b</sup>
$N_{12}P_{24}$	8.15± 0.11 <sup>a</sup>	16.57± 0.10 <sup>cd</sup>	0.85± 0.05 <sup>c</sup>	1.32± 0.02 <sup>a</sup>	8.40± 0.23 <sup>ab</sup>	0.11± 0.01 <sup>cd</sup>	0.32± 0.05 <sup>abc</sup>	11.59± 0.06 <sup>d</sup>	20.84± 0.21 <sup>c</sup>	48.20± 1.25 <sup>b</sup>	14.48± 0.40 <sup>ab</sup>	7.34± 0.37 <sup>a</sup>	33.29± 1.15 <sup>ab</sup>
$N_{24}P_{24}$	7.95± 0.10 <sup>b</sup>	17.01± 0.17 <sup>abc</sup>	0.96± 0.02 <sup>ab</sup>	1.25± 0.04 <sup>b</sup>	8.16± 0.52 <sup>abc</sup>	0.11± 0.01 <sup>cd</sup>	0.32± 0.10 <sup>abc</sup>	13.72± 0.24 <sup>c</sup>	21.06± 0.08 <sup>b</sup>	47.23± 1.16 <sup>b</sup>	14.06± 0.90 <sup>abc</sup>	7.41± 2.01 <sup>a</sup>	35.26± 0.79 <sup>ab</sup>

Note: Different lowercase letters among different treatments indicate significant differences at  $P < 0.05$  level. Mean±SD. TC, total carbon; TN, total nitrogen; TP, total phosphorus; SOC, soil organic carbon; SOP, soil organic phosphorus; SMC, soil moisture content; AP, available phosphorus; SOM, soil organic matter; CK, no fertilizer;  $N_{12}$ , N fertilizer at 90 kg/hm<sup>2</sup>;  $N_{24}$ , N fertilizer at 180 kg/hm<sup>2</sup>;  $P_{12}$ , P fertilizer at 90 kg/hm<sup>2</sup>;  $N_{12}P_{12}$ , N and P fertilizer at 90 and 90 kg/hm<sup>2</sup>, respectively;  $N_{24}P_{12}$ , N and P fertilizer at 180 and 90 kg/hm<sup>2</sup>, respectively;  $P_{24}$ , P fertilizer at 180 kg/hm<sup>2</sup>;  $N_{12}P_{24}$ , N and P fertilizer at 90 and 180 kg/hm<sup>2</sup>, respectively;  $N_{24}P_{24}$ , N and P fertilizer at 180 and 180 kg/hm<sup>2</sup>, respectively. The abbreviations are the same in the following table and figures.

**Table 2** Phospholipid fatty acid (PLFA) content under different nitrogen (N) and phosphorus (P) additions

Treat- ment	Total PLFA (nmol/g)	Bacteria (B) (nmol/g)	Fungi (F) (nmol/g)	F:B ratio	Actinobacteria (nmol/g)	Gram- positive bacteria (GP) (nmol/g)	Gram- negative bacteria (GN) (nmol/g)	GP:GN ratio	Anaerobes (nmol/g)
CK	21.57±3.09 <sup>def</sup>	12.01±1.13 <sup>b</sup>	1.38±0.12 <sup>d</sup>	0.12±0.00 <sup>bc</sup>	2.98±0.25 <sup>b</sup>	5.58±0.57 <sup>de</sup>	7.61±1.72 <sup>cd</sup>	0.75±0.09 <sup>ab</sup>	0.26±0.01 <sup>cd</sup>
N <sub>12</sub>	18.15±1.26 <sup>f</sup>	9.71±0.64 <sup>c</sup>	0.88±0.01 <sup>e</sup>	0.09±0.01 <sup>d</sup>	2.01±0.02 <sup>c</sup>	5.58±0.64 <sup>de</sup>	6.64±1.31 <sup>d</sup>	0.88±0.28 <sup>a</sup>	0.21±0.01 <sup>d</sup>
N <sub>24</sub>	20.11±3.79 <sup>ef</sup>	11.65±2.06 <sup>bc</sup>	1.32±0.28 <sup>d</sup>	0.11±0.00 <sup>c</sup>	2.91±0.39 <sup>b</sup>	4.92±1.06 <sup>c</sup>	7.22±1.18 <sup>cd</sup>	0.68±0.04 <sup>ab</sup>	0.23±0.03 <sup>d</sup>
P <sub>12</sub>	24.52±0.78 <sup>cd</sup>	13.09±0.28 <sup>b</sup>	1.71±0.07 <sup>bc</sup>	0.13±0.00 <sup>a</sup>	2.95±0.17 <sup>b</sup>	5.91±0.12 <sup>cde</sup>	9.41±1.30 <sup>bcd</sup>	0.64±0.10 <sup>b</sup>	0.29±0.01 <sup>cd</sup>
N <sub>12</sub> P <sub>12</sub>	36.87±3.68 <sup>a</sup>	18.26±2.44 <sup>a</sup>	1.74±0.10 <sup>bc</sup>	0.10±0.01 <sup>d</sup>	3.10±0.20 <sup>b</sup>	6.68±0.26 <sup>bc</sup>	19.11±3.94 <sup>a</sup>	0.36±0.07 <sup>c</sup>	0.33±0.03 <sup>bc</sup>
N <sub>24</sub> P <sub>12</sub>	28.11±1.37 <sup>bc</sup>	16.49±0.86 <sup>a</sup>	1.91±0.11 <sup>ab</sup>	0.12±0.00 <sup>bc</sup>	3.71±0.22 <sup>a</sup>	7.25±0.37 <sup>ab</sup>	9.99±0.54 <sup>bc</sup>	0.73±0.00 <sup>ab</sup>	0.35±0.02 <sup>bc</sup>
P <sub>24</sub>	23.49±1.29 <sup>de</sup>	13.06±0.23 <sup>b</sup>	1.64±0.02 <sup>c</sup>	0.13±0.00 <sup>ab</sup>	3.02±0.07 <sup>b</sup>	6.09±0.15 <sup>cd</sup>	8.11±1.11 <sup>bcd</sup>	0.76±0.08 <sup>ab</sup>	0.30±0.02 <sup>bcd</sup>
N <sub>12</sub> P <sub>24</sub>	30.60±1.32 <sup>b</sup>	15.93±0.32 <sup>a</sup>	1.82±0.19 <sup>abc</sup>	0.11±0.01 <sup>bc</sup>	2.79±0.45 <sup>b</sup>	7.76±0.62 <sup>a</sup>	10.98±0.67 <sup>b</sup>	0.71±0.10 <sup>ab</sup>	0.53±0.14 <sup>a</sup>
N <sub>24</sub> P <sub>24</sub>	30.68±2.16 <sup>b</sup>	18.13±1.25 <sup>a</sup>	2.06±0.15 <sup>a</sup>	0.11±0.00 <sup>bc</sup>	4.02±0.30 <sup>a</sup>	7.97±0.58 <sup>a</sup>	11.04±0.72 <sup>b</sup>	0.72±0.01 <sup>ab</sup>	0.39±0.04 <sup>b</sup>

Note: Different lowercase letters among different treatments indicate significant differences at  $P<0.05$  level. Mean±SD.

## 3.2 Changes of soil microorganisms

### 3.2.1 Soil microbial community structure

PLFA content of soil microorganisms after long-term nutrient addition is shown in Table 2. Total soil PLFA was lower in N input treatment than in CK treatment, and total soil PLFA was higher in P addition treatment than in CK treatment; however, the differences were not significant. Total soil PLFA was significantly higher in mixed treatment of N and P than in other treatments ( $P<0.05$ ), with N<sub>12</sub>P<sub>12</sub>, N<sub>24</sub>P<sub>12</sub>, N<sub>12</sub>P<sub>24</sub>, and N<sub>24</sub>P<sub>24</sub> treatments exhibiting 70.93%, 30.32%, 41.86%, and 42.23%, respectively, higher PLFA contents than CK treatment. The trends of bacterial changes in soil with different treatments were consistent with those of total PLFA. Soil actinomycete contents in N<sub>24</sub>P<sub>12</sub> and N<sub>24</sub>P<sub>24</sub> treatments were significantly higher than those in other treatments, with N<sub>24</sub>P<sub>12</sub> and N<sub>24</sub>P<sub>24</sub> exhibiting 24.50% and 34.90%, respectively, higher soil actinomycete content than CK treatment. N<sub>12</sub> treatment had the lowest actinomycete content. Soil anaerobic bacteria levels were significantly higher in N<sub>12</sub>P<sub>24</sub> and N<sub>24</sub>P<sub>24</sub> treatments with 103.85% and 50.00% increase, respectively, compared with soil treated with CK, while soil anaerobic bacteria levels of other treatments were not significantly different from those of CK treatment.

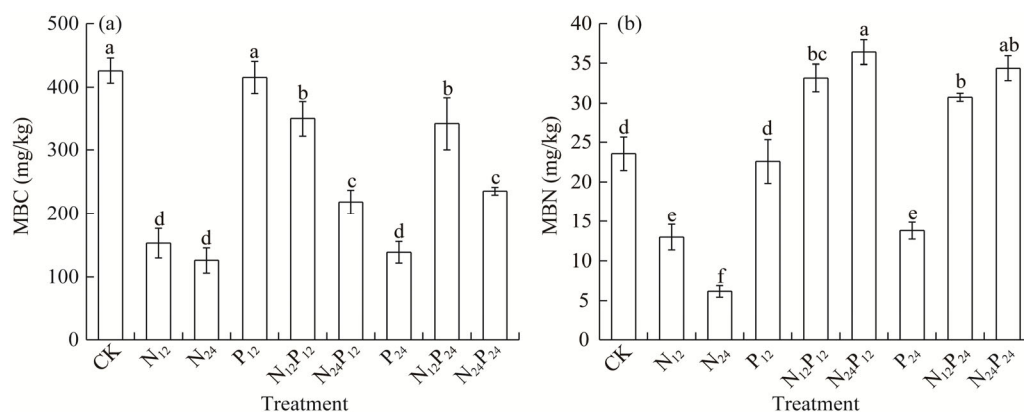
### 3.2.2 Soil microbial biomass

The results of soil microbial biomass analysis after a long-term nutrient addition are shown in Figure 1. Soil MBC content after fertilization was lower than that observed after CK treatment. The highest soil MBC content was 415.32 mg/kg in P<sub>12</sub> treatment and the lowest was 125.52 mg/kg in N<sub>24</sub> treatment (Fig. 1a). Soil MBN content was significantly higher with mixed treatment of N and P than with other treatments, with N<sub>24</sub>P<sub>12</sub> being associated with the highest MBN content, followed by N<sub>24</sub>P<sub>24</sub> and N<sub>24</sub>P<sub>24</sub> (increased by 55.11% and 46.42%, respectively, compared with that of CK treatment). Soil MBN content associated with N fertilizer was significantly lower than that associated with CK treatment, with N<sub>12</sub> and N<sub>24</sub> treatments resulting in decreases by 44.72% and 71.81%, respectively (Fig. 1b).

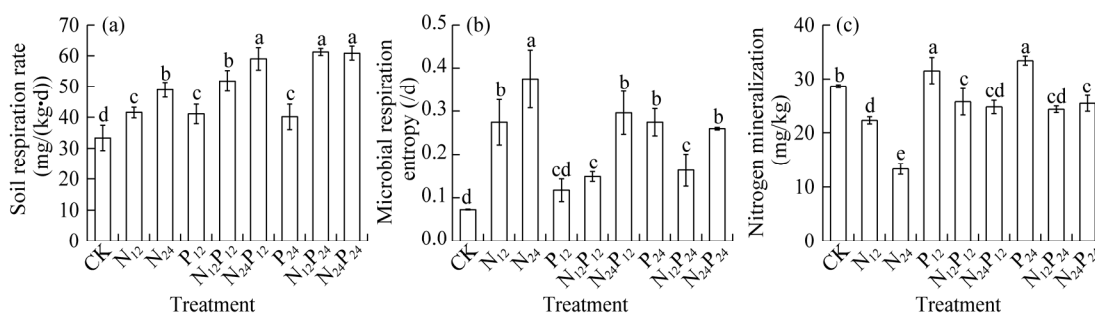
## 3.3 Changes in microbially mediated ecological processes

### 3.3.1 Soil respiration and N mineralization

Long-term nutrient addition had a positive effect on soil respiration levels (Fig. 2a and b). The rate of soil respiration was higher upon mixed treatment of N and P compared with N or P treatment. In addition, fertilization increased soil microbial respiration entropy to varying degrees, which was significantly lower in CK treatment than in other treatments ( $P<0.05$ ), and the highest was 0.37/d with N<sub>24</sub> treatment. The accumulation of N mineralization in the soil fertilized with P (Fig. 2c) was significantly higher than that of CK treatment, N mineralization increasing by



**Fig. 1** Soil microbial biomass carbon (MBC; a) and nitrogen (MBN; b) contents under different nitrogen (N) and phosphorus (P) additions. Different lowercase letters among different treatments indicate significant differences at  $P<0.05$  level. Bars are standard errors.



**Fig. 2** Soil respiration rate (a), microbial respiration entropy (b), and nitrogen mineralization (c) under different nitrogen (N) and phosphorus (P) additions. Different lowercase letters among different treatments indicate significant differences at  $P<0.05$  level. Bars are standard errors.

10.21% and 16.89% in P<sub>12</sub> and P<sub>24</sub> treatments, respectively. In contrast, application of N fertilizer had a significant inhibitory effect on N mineralization, which decreased with increasing N treatment, N mineralization decreasing by 22.04% and 53.45% in N<sub>12</sub> and N<sub>24</sub> treatments, respectively. The level of N mineralization in soils with mixed treatment of N and P was lower than that with CK treatment.

### 3.3.2 Soil enzyme activity

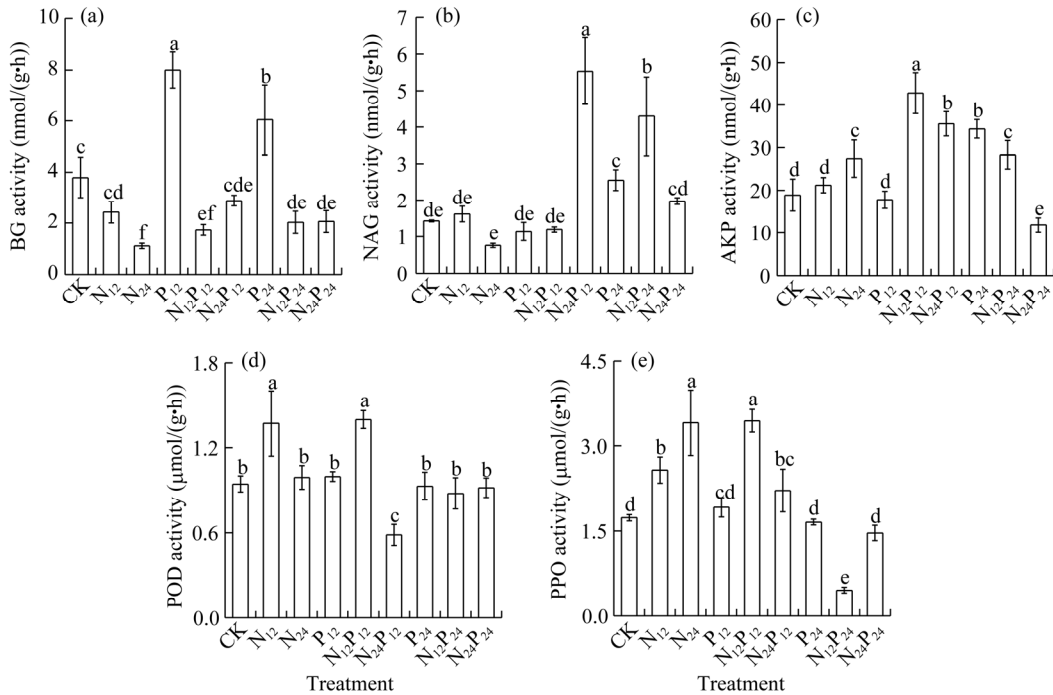
As shown in Figure 3, BG activity was significantly increased by P addition, with BG activity increasing by 111.08% and 59.63% in P<sub>12</sub> and P<sub>24</sub> treatments, respectively, compared with CK treatment (Fig. 3a). N<sub>24</sub> treatment had the lowest activity (0.10 nmol/(g·h)). NAG activity was the highest in N<sub>24</sub>P<sub>12</sub> treatment (5.54 nmol/(g·h)), followed by N<sub>12</sub>P<sub>24</sub> treatment (4.30 nmol/(g·h)), and was the lowest in N<sub>24</sub> treatment (46.48% reduction compared with CK treatment, Fig. 3b). Under conditions of mixed treatment of N and P, AKP activity decreased with increasing concentration of fertilizer. The highest activity was observed in N<sub>12</sub>P<sub>12</sub> treatment (42.83 nmol/(g·h)), and the lowest activity was observed in N<sub>24</sub>P<sub>24</sub> treatment (11.75 nmol/(g·h)) (Fig. 3c).

N<sub>12</sub> and N<sub>12</sub>P<sub>12</sub> treatments significantly increased the activities of two soil oxidases, i.e., PPO and POD, by 49.13% and 98.84%, and 44.21% and 47.37%, respectively, compared with CK treatment. PPO activity was the lowest (0.46  $\mu$ mol/(g·h)) in N<sub>12</sub>P<sub>24</sub> treatment (Fig. 3d). POD activity in N<sub>24</sub>P<sub>12</sub> treatment was significantly lower (38.95%) than that in CK treatment, and the differences in enzymatic activities in response to other treatments were not significant (Fig. 3e).

## 3.4 Environmental factors driving soil microbial community structure and enzymatic activity

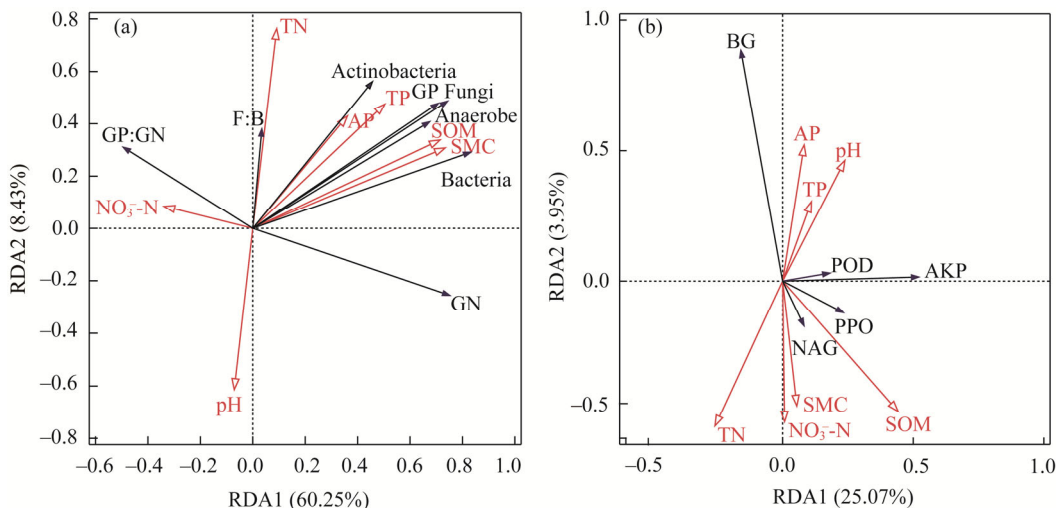
### 3.4.1 Correlation analysis

RDA based on the relationship between soil environmental factors and microbial PLFA content



**Fig. 3** Activities of BG ( $\beta$ -1,4-glucosidase, a), NAG ( $\beta$ -N-acetylglucosaminidase, b), AKP (alkaline phosphatase, c), POD (peroxidase, d), and PPO (polyphenol oxidase, e) under different nitrogen (N) and phosphorus (P) additions. Different lowercase letters among different treatments indicate significant differences at  $P < 0.05$  level. Bars are standard errors.

with different N and P additions (Fig. 4a) showed that soil physical-chemical properties could explain 68.68% of changes in soil microbial community structure. SMC, TP, and TN were the most important factors, explaining 33.30%, 16.00%, and 6.20% of the variations, respectively. Bacteria, fungi, Actinobacteria, GP bacteria, anaerobes, and F:B ratio were positively correlated

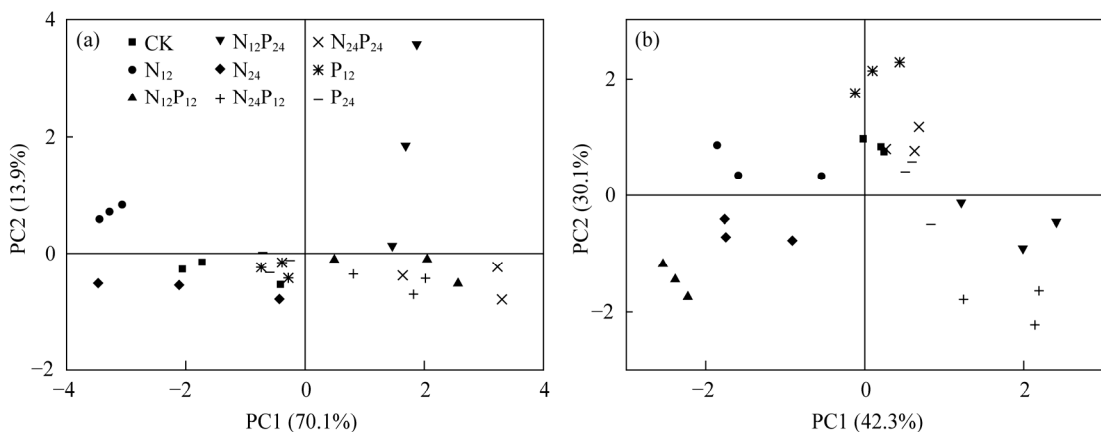


**Fig. 4** Redundancy analysis (RDA) of microbial community structure (a) and enzyme activity (b) with soil physical-chemical properties. Red arrows represent soil property variables, and black arrows denote soil microbial taxa (a) and activities of five enzymes (b). TN, total nitrogen; TP, total phosphorus; AP, available phosphorous; SOM, soil organic matter; SMC, soil moisture content; GP, gram-positive bacteria; GN, gram-negative bacteria; GP:GN, gram-positive bacteria:gram-negative bacteria ratio; F:B, fungal:bacterial ratio; BG,  $\beta$ -1,4-glucosidase; POD, peroxidase; AKP, alkaline phosphatase; PPO, polyphenol oxidase; NAG,  $\beta$ -N-acetylglucosaminidase.

with SOM, SMC, TP, AP, and TN, and negatively correlated with pH. RDA was performed using five enzyme activities as response variables and soil environmental factors as explanatory variables (Fig. 4b). Results revealed that the two axes together explained 29.02% of the variation in soil enzyme activity, and TN was the environmental factor with the highest degree of explanation (14.20%). NAG was positively correlated with SOM, SMC, TN, and  $\text{NO}_3^-$ -N, and negatively correlated with AP, TP, and pH. BG and AKP were positively correlated with TP, AP, and pH, and negatively correlated with TN and  $\text{NO}_3^-$ -N.

### 3.4.2 Principal component analysis (PCA)

PCA of soil microbial PLFA showed (Fig. 5a) that soil microbial community structure was different, with PC1 (principal component 1) and PC2 (principal component 2) axes explaining 70.1% and 13.9% of the differences, respectively. The corresponding points of N and P additions and CK treatment were projected in the negative direction of PC1, indicating that soil microbial community structure was similar in these three treatments. The corresponding points of four treatments with N and P additions were projected in the positive direction of PC1 and showed a positive correlation, indicating that N and P additions had a greater impact on soil microbial community structure. PCA of soil enzyme activity (Fig. 5b) revealed that  $\text{P}_{12}$ ,  $\text{P}_{24}$ , and  $\text{N}_{24}\text{P}_{24}$  treatments were close to CK treatment, indicating that these three treatments did not have a significant effect on the overall soil enzyme activity, while N fertilizer,  $\text{N}_{12}\text{P}_{12}$ , and  $\text{N}_{24}\text{P}_{12}$  treatments had a greater effect on the overall soil enzyme activity.



**Fig. 5** Principal component analysis (PCA) of microbial phospholipid fatty acid (PLFA; a) and soil enzyme activities (b). PC, principal component.

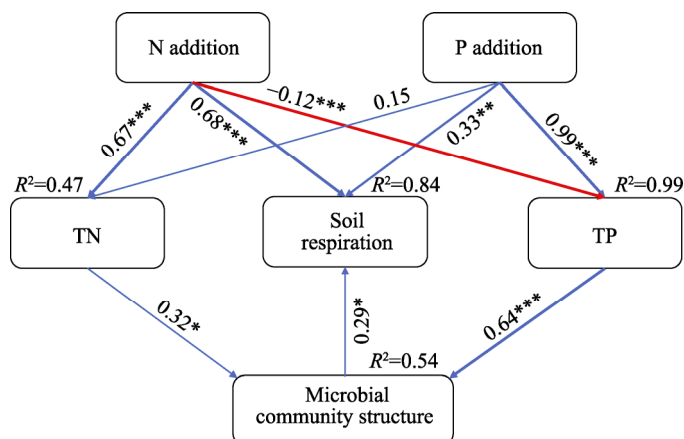
## 3.5 Changes in soil microbial community structure and ecological processes

### 3.5.1 Environmental factors driving changes in microbial community structure

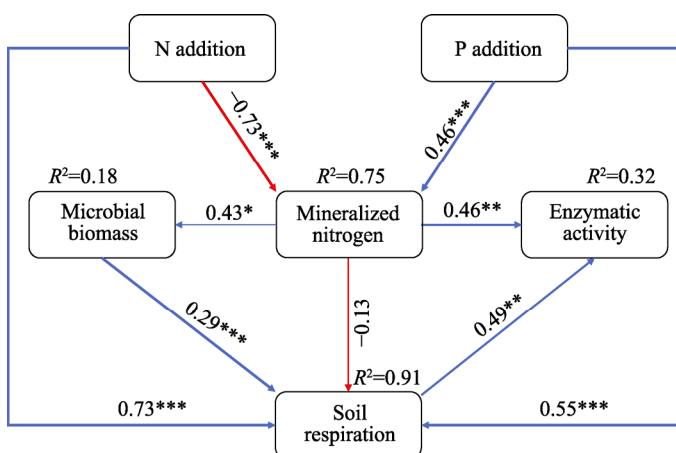
RDA demonstrated that soil physical-chemical properties (SMC, TP, and TN) dominated the changes in the microbial community structure (Fig. 4a). Therefore, we further quantified the effect of N and P additions on the microbial community structure by altering soil nutrient content using a structural equation model (SEM; Fig. 6). The model indicated that soil TN and TP contents together explained 54.0% of the total variation in microbial community structure. In addition, soil respiration was directly affected by N and P additions and changes in microbial communities, which together explained 84.0% of the total variations in soil respiration.

### 3.5.2 Links between ecological processes

SEM of the effect of N and P additions on microbially mediated ecological processes showed (Fig. 7) that N and P additions along with the microbial biomass directly contributed to soil respiration rate, with three together explaining 91.0% of the total variations in soil respiration. N and P additions together explained 75.0% of the accumulated soil N mineralization, whereas N addition indirectly affected microbial biomass and soil enzyme activity by inhibiting N mineralization.



**Fig. 6** Structural equation model (SEM) of the effect of nitrogen (N) and phosphorus (P) additions on microbial community structure. Soil microbial community structure in the model was scored by PC1 (principal component). The final model had good fit to the data, with model  $\chi^2=5.566$ ,  $df=6$ ,  $P=0.474$ , root mean square error of approximation (RMSEA)=0.000 and comparative fit index (CFI)=1.000. Blue and red arrows indicate positive and negative relationships, width of the arrow is proportional to the positive and negative relationships, and value on the arrow indicates the normalized path coefficient. Asterisks following the values indicate statistical significance of paths, \*,  $P<0.05$  level, \*\*,  $P<0.01$  level, \*\*\*,  $P<0.001$  level.



**Fig. 7** Structural equation model (SEM) of the effects of nitrogen (N) and phosphorus (P) additions on soil ecological processes. Soil enzyme activities in the model were scored by PC1 (principal component). The final model resulted in good fit to the data, with model  $\chi^2=3.254$ ,  $df=6$ ,  $P=0.776$ , root mean square error of approximation (RMSEA)=0.000 and comparative fit index (CFI)=1.000. Blue and red arrows indicate positive and negative relationships, respectively. Width of the arrow is proportional to the positive and negative relationships, and value on the arrow indicates the normalized path coefficient. Asterisks following the values indicate statistical significance of paths, \*,  $P<0.05$  level, \*\*,  $P<0.01$  level, \*\*\*,  $P<0.001$  level.

## 4 Discussion

### 4.1 Changes in microbial community structure

Microbial biomass and community structure play critical roles in soil fertility and stability. We found that the application of N and P fertilizers did not have a significant effect on the total PLFA content of the soil (Table 2; Fig. 5a). Meta-analysis revealed a lack of response of the total PLFA content to N addition (Zhu et al., 2016). The positive effects on microbial growth by increasing litter input and N supply may be offset by the negative effects of soil acidification and metal toxicity (Treseder, 2008; Tian and Niu, 2015), resulting in no change in total PLFA.

Soil pH is a key factor in altering the structure of microbial community as long-term N

application treatments lead to soil acidification, which has a negative effect on bacterial and fungal contents (Dai et al., 2018; Zhang et al., 2018; Wang et al., 2020). In this study, application of low N fertilizer had no effect on soil pH, however, it significantly reduced soil bacterial and fungal contents, whereas application of high N fertilizer significantly reduced soil pH, but did not change bacterial and fungal contents (Tables 1 and 2). The impact of N-induced soil acidification is contingent upon various factors, including the intensity of fertilizer application, soil buffering capacity, and land use history (Ma et al., 2023), as well as the potential soil-specific responses of bacterial and fungal communities to fertilizer application (Liu et al., 2018). Consequently, in contrast to previous studies, alterations observed in the microbial community structure within this investigation may be associated with other environmental factors rather than pH. RDA (Fig. 4b) and SEM (Fig. 6) showed that under nutrient addition conditions, the contents of TP and TN in the soil jointly dominated the changes in microbial community structure, indicating a close relationship between soil nutrient pools and microorganisms. And the result is same as our first hypothesis.

Compared with only N addition, total PLFA, bacterial, and fungal contents significantly increased when combined with P addition (Table 2; Fig. 5a), indicating a significant positive interaction effect of N and P, which is consistent with previous studies (Guo et al., 2017). Total PLFA, bacteria, fungi, actinomycetes, and GN bacteria were significantly higher in soils with long-term application of N and P fertilizers than in soils with only N fertilizers. The increased levels of bacteria, fungi, and anaerobic bacteria in the soil may be due to the fact that the input P sources increased the amount of AP in the soil and alleviated the strict P limitation in the ecosystem of the region, thus, indirectly affecting microbial activity (Wang et al., 2022), and the increased nutrients available to soil microorganisms after N and P fertilizers directly promoted the growth and reproduction of soil microorganisms (Wang et al., 2017). Thus, we concluded that soil microbial community in the study area was affected by common limitations of N and P. When only P or N was added, the limitations of other nutrients remained, although the availability of added element increased. However, when they were co-injected, the restrictive effects of P and N were simultaneously alleviated, thereby stimulating microbial growth.

#### 4.2 Changes in soil biotic and abiotic parameters

Our study showed that long-term N addition decreased TP and AP contents in the soil (Table 1), which could be attributed to the accelerated demand for P in crops (Fisk et al., 2014), and with the application of crops rotation, P in the soil is in a state of deficit, resulting in a decrease in its activation ability (Wang et al., 2018). Moreover, N significantly reduced soil MBC and MBN contents (Fig. 1) and mineralized N accumulation (Fig. 2c) due to N-induced loss of associated basic cations and C limitation, inhibiting potential N saturation of  $\beta$ -glucosidase activity in the soil and resulting in reduced microbial C acquisition and biomass. When available N is sufficiently abundant in the soil, microorganisms no longer mineralize organic matter to obtain available N resources (Wang et al., 2020). The availability of P affects N mineralization by mediating microbial activity (Bicharanloo et al., 2022), and our study revealed that the addition of P significantly increased the accumulation of N mineralization. The balance between mineralization and immobilization accelerates N mineralization in the soil (Wang et al., 2022). Consistent with our second hypothesis, there was no significant difference in soil N mineralization among four treatments with mixed treatment of N and P, but all were lower than CK treatment and higher than N treatment (Fig. 2c), probably because inorganic N content of the soil was in a saturated state, resulting in greater N inhibition than P promotion.

N addition does not always have a negative impact on the ecological functions of the soil. We found that N fertilizer treatment increased PPO and POD activities (Fig. 3d and e), which can be explained by the "resource allocation theory" (Allison and Vitousek, 2005). This theory predicts that elevated N availability increases the microbial demand for C, inducing the production of C-acquiring enzymes (Huang et al., 2018). Changes in the soil physical-chemical properties drove changes in soil enzyme activities under N and P additions, such as pH and total available N and P contents (Fig. 4b). These findings suggest that nutrients in the soil stimulate enzyme synthesis to

break down most organic compounds and soil microbial community (bacterial, fungal, and actinomycete PLFA content) increases in response to improvements in the physical structure and chemical properties of the soil, promoting the ability of microorganisms to synthesize enzymes (Cui et al., 2019).

Ecological effects of N and P additions on soils are not always opposing, primarily manifesting as a synergistic effect on soil respiration (Fig. 2a). N enrichment stimulates plant growth by increasing substrate input into the soil and promoting soil respiration (Chen et al., 2017). AP in the soil significantly increases soil respiration by reducing microbial nutrient limitation and stimulating root growth (Lu et al., 2022), whereas P addition directly increases soil P availability. Significantly, mixed treatment of N and P has been found to alter the magnitude of the effects exerted by N or P individually on soil respiration. This result confirms our third hypothesis that the interaction between these two nutrients enhances the positive effect on soil respiration. The influence of mixed treatment of N and P on soil respiration response is partially contingent upon P. The addition of P mitigates the inhibitory effects of litter-fall accumulation on soil respiration, while concurrently enhancing leaf N content and promoting N use efficiency through improved soil N availability (Liu et al., 2021). These dual strategies serve to amplify the positive effects of N addition on soil respiration.

### 4.3 Relationships between soil ecological processes

Soil is an important natural reservoir of C and N, and the ecological function of soil is a key process in the material cycle and energy exchange of terrestrial ecosystems. The process of N mineralization in forest soils is known to be influenced by several factors, either individually or through interactions, including microbial activity, microbial extracellular enzymes, and soil C:N ratio (Gao et al., 2015). However, our study reveals a previously overlooked aspect, namely that mineralized N also plays a regulatory role in soil microbial and enzymatic activities. Our findings indicate that N mineralization significantly affects soil microbial activity (Fig. 6), including microbial biomass and respiration, as well as enzymatic activity. In contrast to forest ecosystems, the soil heterotrophs within artificially cultivated environments face limited nutrient acquisition opportunities due to competition from crops, as well as diminished C metabolic efficiency (Kaye and Hart, 1997). Consequently, a greater amount of SOM must undergo decomposition to meet the nutrient demands of these organisms. N mineralization, in particular, influences C:N ratio of soil, consequently impacting the nutrient uptake capabilities of microorganisms. Soil MBN content reflects the effect of soil microorganisms on N mineralization and fixation. Any factors that affect these two processes change MBN content, thereby changing soil microbial biomass and synthesis of enzymes needed to consume N. Therefore, the level of N mineralization affects the overall enzyme activity of the soil. Yan et al. (2021) discovered that excessive N input negatively affected soil microbial communities and biomass in agricultural fields, forming undegradable compounds that reduce the mineralization activity of soil microorganisms and inhibit the activity of BG. This explains the negative effects of N addition on soil microbe-mediated ecological processes in our study (Figs. 1, 2c, and 3a) and demonstrates that the study area is susceptible to P limitation.

Microbial biomass, enzyme activity, and soil respiration varied with long-time of nutrient additions. Previous studies found that N and P additions did not affect microbial community structure and elemental cycling (Jing et al., 2020; Ma et al., 2020). This difference may be due to multiple factors. Soil microorganisms may face distinct resource limitations based on ecosystem types. For forest ecosystems, high N levels are typically exhibited, while C and P are often limiting factors (Li et al., 2015). Furthermore, the duration of nutrient addition is also an important factor in the change of soil ecological function. Compared with short-term nutrient addition (Ma et al., 2020), the duration of nutrient input in this study is as long as 36 a, which is also the innovation of this study. However, soil microbial community structure and function may vary by season and soil depth. Therefore, sampling at different time points and soil depths is needed to fully reveal the response of soil microorganisms to nutrient addition in the semi-arid area of loess.

## 5 Conclusions

Long-term N and P additions significantly increased total PLFA, bacterial and fungal PLFA contents, changed microbial community structure, and increased soil respiration rate and microbial biomass in agricultural soils in loess areas. N addition had a negative effect on soil microbial biomass, BG activity, and mineralized N accumulation, while P addition was able to mitigate or modify this negative effect. Soil microbial community structure and ecological processes are driven by different environmental mechanisms, with soil nutrient pool (TN and TP) content dominating changes in microbial community structure and thus affecting soil respiration, while changes in soil enzyme activity are mediated by soil available nutrients ( $\text{NO}_3^-$ -N and AP) and N mineralization. In conclusion, P addition and mixed addition N and P had positive impacts on soil microbial characteristics, ecological functions, and soil productivity. Our study provides a scientific basis that enables local farmers to choose a good fertilizer application program, thereby positively affecting the sustainable development of the environment in the Loess Plateau.

## Conflict of 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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## Author contributions

Conceptualization: LI Chunyue, KOU Zhaoyang, DANG Tinghui; Methodology: KOU Zhaoyang, CHANG Shun, MIAO Yu, ZHANG Wenting, LI Qianxue; Formal analysis: LI Chunyue, KOU Zhaoyang, CHANG Shun, MIAO Yu, LI Qianxue; Writing - original draft preparation: KOU Zhaoyang; Writing - review and editing: KOU Zhaoyang, LI Chunyue, WANG Yi, ZHANG Wenting; Funding acquisition: LI Chunyue.

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