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Soil microbes influence nitrogen limit on the plant biomass of the alpine steppe, North Tibet

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Research Article

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Abstract

It is uncertain that plant biomass of grassland was co-limited by nitrogen (N) and phosphorus (P) or only limited by N or P. Moreover, it is also unclear why plant biomass was not limited by the P when the N was added at grassland ecosystem. The N and P additional experiment was conducted at the alpine steppe from 2013 to 2017. Results from analyzing response ratio of shoot biomass and leaf N and P concentration to N and P addition suggested that N is the main limiting factor for plant biomass of alpine steppe, and P is synergistic with N in contributing to biomass. Based on the N:P stoichiometry, plants have to increase P absorption due to N addition. Results of SEM analysis showed plant and fungi promoted the activity of phosphatase. At the same time, the abundance of fungi and saprotrophic group associated with decomposition were increased due to N addition, which would provide more P for plant. Those results suggested P deficit of plant due to N addition would be alleviated. Similarly, plant have to increase the N absorption when P was added. Although activity of urease was enhanced by soil microbes and plant, there was a little substrate for microbes because plant biomass was not increased due to P addition. Therefore, composition and function of soil microbes were affected by plant and soil N:P due to N addition, and then delivered P for plant, which will influence the effect of N on plant biomass at alpine steppe.

Introduction

The grassland ecosystem is one of the most important ecosystem,which remains uncertain whether nitrogen (N) and phosphorus (P) have co-limited or synergistic effects on plant biomass. Elser et al. (2007) suggested that N and P have a co-limited effect on biomass based on a meta-analysis of experimental enrichments. Bracken et al. (2015) suggested N and P co-limited the plant biomass through analyzing the nutrient concentrations. However, LeBauer and Treseder (2008) suggested that N limited the biomass of grassland, and Fay et al. (2015) suggested N was the main biomass-limiting nutrient in cool, high-latitude grassland sites. However, according to ecological stoichiometry theory, plant growth was co-limited by N and P. Therefore, it is unclear why the plant biomass of grassland ecosystem was not limited by P when N was added.

It is well known that soil microbes play crucial roles in several key biogeochemical processes (Nannipieri et al. 2003; Fierer 2017) that are essential for soil fertility and plant growth (Kennedy 1999; Artursson et al. 2006). Moreover, interactions among microbes have a potential influence on soil nutrient cycling and plant health (Johansson et al.2004; Huang et al. 2019), which could influence the effect of N and P on the biomass in the grassland ecosystem (Van Der Heijden et al. 2008). Therefore, the non-determinacy conclusion that plant biomass limited by the N or P maybe correlated with changing in function of soil microbial community.

The grassland ecosystem is experiencing N deposition due to anthropogenic emissions of reactive nitrogen (Liu et al. 2013; Stevens et al. 2016). Increasing N deposition increases the content of active N in soil, which then correspondingly increases the biomass and also alters the composition of the plant and

microbial communities (Wang et al. 2010; Wang et al. 2012; Tian et al. 2014; He et al. 2016; Xiang et al. 2016). However, it remains unknown whether the plant biomass is limited by P when the N content increases. Moreover, few studies have focused on whether the co-limited or synergistic effect of N and P on the biomass is influenced by soil microbes. In order to understand these processes, we conducted an N and P addition experiment over a 5-year period on the alpine steppe of North Tibet. The aim of present study was to determine (1) whether P co-limits biomass with N or has a synergistic effect, and (2) how microbes influence the effect of N and P on plant biomass at grassland ecosystem.

Materials And Methods

Study sites and experimental design

The experiment was carried out at the Xianzha Alpine Steppe and Wetland Ecosystem Observation and Experiment Station on the northern Tibetan Plateau (30°57'N, 88°42'E, 4675 m a.s.l). *Stipa purpurea* and *Carex moorcroftii* are the dominant species, and *Artemisia nanschanica* is a companion species. The soils consist of frigid calcic soils and seasonally frozen soil. The sand content of the soil is about 77 percent and the soil pH is about 8. The content of soil available N is 42.88 mg kg^{-1} and the content of soil available P is 1.76 mg kg^{-1}. The experiment included a control treatment (CK) and N addition (N), P addition (P), and combination of N and P addition (N + P). Twelve plots (4 × 4 m) were established and were separated by 1 m intervals. The N addition rate was 100 kg ha^{-2} yr^{-1} (NH_4NO_3) and the P addition rate was 40 kg ha^{-2} yr^{-1} (K_2HPO_4 and KH_2PO_4 were selected, which were mixed to maintain a pH value of 7. The N, P and N + P amendments were applied twice (at the beginning of June and July from 2013 to 2017). The N, P and N + P fertilizers were dissolved in 1 L of water and sprayed evenly over each plot.

Sampling

Plant and soil were collected in mid-August 2017. The methods are as same as that of Wu et al. (2020).

Real-time PCR and MiSeq sequencing

Soil microbial DNA was extracted using an Ezup genomic DNA extraction kit for soil (Sangon Biotech, China, Cat# SK8264). Soil microbial DNA concentrations and quality were checked by a NanoDrop Spectrophotometer. Fungal and bacterial abundances were calculated by qRT-PCR (CFX 384 Real-Time system,Bio-Rad) according to Wu et al (2020).

Soil properties

The soil pH was estimated using a pH meter (E20-FiveEasyTM pH, Mettler Toledo, German) in a 1:5 soil:deionized water (wt/v) ratio. Available P (AP) was extracted via sodium bicarbonate solution and determined using the molybdenum blue method (Lu, 1999). Total organic carbon (TOC) and dissolved organic carbon (DOC) was estimated with a TOC analyzer (Multi N/C 3000, Analytik Jena, Germany). Total N (TN) were determined using an elemental analyzer (Vario Max Elementar, Germany). Available N (AN) was estimated using the alkaline hydrolysis method (Lu,1999). NO_3-N and NH_4-N were extracted

with 1 mol L^{-1} KCl and then determined using a Foss FIAstar 5000 flow injection autoanalyzer. The total dissolved N (TDN) was extracted with 2 mol L^{-1} KCl and then measured with a TOC-TN analyzer (Shimadzu, Kyoto, Japan). The DON was calculated as follows: DON = TDN − NH_4-N − NO_3-N. .Total phosphorus (TP) was determined using soil determination of total phosphorus by the alkali fusion Mo-Sb anti spectrophotometric method (Lu 1999). Soil urease (UE) and phosphatase (NEP) were measured with a soil UE ELISA kit and soil NEP ELISA kit (Jiangsu, China), respectively.

Leaf N and P contents

There are about 6 species per meter square in the experiment site. So, we select the dominant species (*S. purpurea* and *C. moorcroftii*) and main companion species (*A. nanschanica*), which shoot biomass is about 80% of total shoot biomass. The leaf N concentration was measured by the semi-micro Kjeldahl method (Lu 1999). The leaf P concentration was measured by the alkali fusion Mo-Sb anti spectrophotometric method (Lu 1999).

Composition and function of bacteria and fungi data analysis

The sequence data of bacteria and fungi were processed using QIIME Pipeline (http://qiime.org/). Composition of fungal and bacterial community was calculated according to Wang et al.(2007). Biogeochemical cycle functional annotation of the bacterial taxa was performed using the program FAPROTAX (version 1.1) based on the normalized OTU table (Louca et al. 2016). Trophic functional annotation of fungal taxa was separately analyzed using FUNGuild based on the normalized OTU table (Nguyen et al. 2016).

Statistical analysis

The differences in soil properties, plant properties and microbial abundance among different N and P addition treatments were tested by one-way ANOVA followed by a Duncan's test ($p < 0.05$). Two-way ANOVA was followed by a Duncan's test for multiple comparisons, which checked three aspects (N effect, P effect and N + P effect) for soil microbes and plant community characteristics. The response ratio was used to define whether the biomass was limited by N or P (Elser et al. 2007; LeBauer and Treseder 2008;Fay et al. 2015; Bracken et al. 2015). The response ratio $R_{biomass}$: R_B =shoot $biomass_{(fertilization)}$ / shoot $biomass_{(control)}$. The response ratio $R_{N\ or\ P}$: $R_{N\ or\ P}$ =N or P concentration of leaf $_{(fertilization)}$/N or P concentration of $leaf_{(control)}$. All analysis procedures were executed in SPSS 21.0 (SPSS, Chicago, IL, USA). Structural equation model (SEM) was conducted to estimate the effects of N and P addition on plant biomass, soil N:P, microbes, phosphatase and urease. The maximum likelihood calculation was used to fit the model by using the IBM SPSS-Amos 26.0.

Results

Response ratio (R) of shoot biomass and leaf N and P concentration to N and P additions

The response ratio of shoot biomass (R_B) in N and N + P treatment was positive effect ($p < 0.05$) (Table 1), but it was no significant effect in P treatment ($p > 0.05$) (Table 1). The response ratio leaf N concentration (R_N) of three species (*S.purpurea, C.moorcroftii and A.nanschanica*) in N and N + P treatment were positive effect ($p < 0.05$), but they were no significant effect in P treatment ($p > 0.05$) (Table 1). The response ratio leaf P concentration (R_P) of three species in P and N + P treatment were positive effect ($p < 0.05$), but they were no significant effect in N treatment ($p > 0.05$) (Table 1).

Table 1
Responses ratio (R) of shoot biomass, leaf nitrogen and phosphorus concentration to N, P, and N + P treatment.

Response ratio (R)		**Treatment**		
		N	**N + P**	**P**
Shoot biomass (R_B)		+	+	0
N concentration of leaf (R_N)	*S.purpurea*	+	+	0
	C.moorcroftii	+	+	0
	A.nanschanica	+	+	0
P concentration of leaf (R_P)	*S. purpurea*	0	+	+
	C. moorcroftii	0	+	+
	A.nanschanica	0	+	+

Symbols indicate statistically significant ($p < 0.05$) positive (+) and negative (–) effects relative to controls, and 0 denotes no significant effect of nutrient additions.

For the shoot biomass, N and N + P additions produce higher responses ratio of shoot biomass than P additions ($p < 0.05$; Table 2). There is not significant difference between N and N + P treatment ($p > 0.05$; Table 2). For the N concentration of leaf, the R_N of three species in N and N + P treatment are significantly higher than those in P treatment ($p > 0.05$; Table 2). There is also not significant difference between N and N + P treatment ($p > 0.05$; Table 2). For the P concentration of leaf, the R_P of three species in N + P and P treatment are significantly higher than those in N treatment ($p > 0.05$; Table 2). There is also not significant difference between P and N + P treatment ($p > 0.05$; Table 2).

Table 2
Results of ANOVA's comparing the responses ratio (R) of the three nutrient enrichment treatments (N, P, and N + P) on shoot biomass and N, P concentration of leaf

Response ratio (R)		Treatment	d.f.	Sum of squares	F	*p*-value
Shoot biomass(R_B)		N vs N + P	1	0.170	0.262	0.636
		N + P vs P	1	0.727	14.828	**0.018**
		N vs P	1	0.986	16.058	**0.016**
N concentration of leaf (R_N)	*S.purpurea*	N vs N + P	1	0.003	0.127	0.740
		N + P vs P	1	0.810	39.613	**0.003**
		N vs P	1	0.904	105.079	**0.001**
	C.moorcroftii	N vs N + P	1	0.000	0.002	0.969
		N + P vs P	1	0.369	10.337	**0.032**
		N vs P	1	0.332	9.753	**0.035**
	A.nanschanica	N vs N + P	1	0.014	0.176	0.696
		N + P vs P	1	0.374	25.229	**0.007**
		N vs P	1	0.532	8.115	**0.046**
P concentration of leaf (R_P)	*S. purpurea*	N vs N + P	1	2.068	10.918	**0.030**
		N + P vs P	1	0.278	1.395	0.303
		N vs P	1	0.830	41.760	**0.030**
	C. moorcroftii	N vs N + P	1	0.604	18.638	**0.012**
		N + P vs P	1	0.001	0.006	0.942
		N vs P	1	0.573	11.147	**0.029**
	A.nanschanica	N vs N + P	1	1.614	44.573	**0.003**
		N + P vs P	1	0.084	2.557	0.185
		N vs P	1	0.962	83.903	**0.001**

Influence of N and P addition on soil properties

Soil properties were significantly affected by N and P addition after 5 years (Table 3). Soil pH and TOC were not significantly affected by N and P addition ($p > 0.05$). The DOC decreased following the N treatment ($p < 0.05$). The TN, AN, DON, NO_3-N and NH_4-N increased significantly in the N and N + P treatments ($p < 0.05$). There were no significant difference in TN, AN, NO_3-N and NH_4-N between the N and

N + P treatments ($p > 0.05$). NH_4-N decreased significantly in the P treatment ($p < 0.05$). TP and AP were significantly enhanced in the P and N + P treatment ($p < 0.05$), but were not significantly affected in the N treatment ($p < 0.05$). The activities of soil UE, NEP and AP were significantly promoted following N and P addition ($p < 0.05$). There was no significant difference in the activity of UE and NEP between the N treatment and N + P treatment ($p > 0.05$). However, the activity of AP in the N treatment is significantly higher than that in other treatments (Table 3).

Table 3
Effects of N and P addition on soil physicochemical property (mean ± s.e)

Treatment Variables	CK	N	N + P	P
pH	8.22 ± 0.01a	8.25 ± 0.04a	8.26 ± 0.05a	8.32 ± 0.01a
TN (g kg^{-1})	1.45 ± 0.07b	1.72 ± 0.10a	1.94 ± 0.09a	1.39 ± 0.10b
AN (mg kg^{-1})	13.61 ± 0.29c	31.08 ± 2.34a	26.80 ± 0.84b	14.28 ± 0.84c
DON (mg kg^{-1})	4.68 ± 0.31c	9.83 ± 0.76b	20.24 ± 2.56a	12.28 ± 2.53b
NO_3-N (mg kg^{-1})	16.74 ± 1.18b	28.30 ± 1.44a	29.54 ± 1.86a	17.04 ± 2.69b
NH_4-N (mg kg^{-1})	3.64 ± 0.21b	4.34 ± 0.12a	4.31 ± 0.22a	2.95 ± 0.22c
STP (g kg^{-1})	0.43 ± 0.01b	0.41 ± 0.01b	0.55 ± 0.02a	0.51 ± 0.02a
SAP (mg kg^{-1})	2.16 ± 0.18b	2.07 ± 0.08b	15.77 ± 1.49a	15.45 ± 4.85a
UE (U g^{-1})	350.92 ± 11.86c	536.23 ± 14.00a	568.06 ± 12.98a	421.51 ± 7.29b
NEP (U g^{-1})	0.42 ± 0.01c	0.66 ± 0.02a	0.66 ± 0.01a	0.49 ± 0.02b
AP (U g^{-1})	1.60 ± 0.06b	2.57 ± 0.13a	1.50 ± 0.08b	1.33 ± 0.10b

TOC, total organic carbon; DOC, dissolved organic carbon; TN, total nitrogen; AN, available nitrogen; DON, dissolved organic nitrogen; NO_3-N, nitrate-nitrogen; NH_4-N, ammonium nitrogen; TC, total carbon; STP, soil total phosphate; SAP, soil available phosphate; UE, urease; NEP, neutral phosphatase; AP, acid phosphatase. The different letter in the same row indicate significant difference at the 0.05 level.

Response of the microbial community composition to N and P addition

The composition of the bacterial community was not significantly influenced by the N factor; however, it was significantly affected by the P factor (Table 4, Fig. 1A). Thaumarchaeota were also significantly affected by the interactive effect of the N and P factors (F = 6.406, $p < 0.05$). The composition of the fungal community was significantly influenced by N and P addition (Table 4, Fig. 1B). Ascomycota were

significantly affected by the N factor and the interactive effect of the N and P factors (F = 13.484, $p < 0.01$; F = 7.321, $p < 0.05$). Mortierellomycota were significantly affected by N and P addition (F = 9.767, $p < 0.05$;F = 12.089, $p < 0.01$;F = 12.866, $p < 0.01$). Basidiomycota were significantly affected by P addition (F = 5.836, $p < 0.05$).

Table 4
Specific and interactive effects of N and P factors on the relative abundance of bacterial and fungal phyla among different treatments

		N factor		**P factor**		**N×P**	
Variables		F **value**	p **value**	F **value**	p **value**	F **value**	p **value**
Bacterial phylum	Thaumarchaeota	3.668	0.092	15.8	**0.004**	6.406	**0.035**
	Actinobacteria	3.132	0.115	13.156	**0.007**	4.452	0.068
	Proteobacteria	2.285	0.169	9.15	**0.016**	3.397	0.103
	Acidobacteria	3.863	0.085	13.466	**0.006**	5.128	0.053
	Chloroflexi	2.854	0.130	7.659	**0.024**	3.946	0.082
	Bacteroidetes	2.497	0.153	7.981	**0.022**	2.562	0.148
	Gemmatimonadetes	2.901	0.127	8.737	**0.018**	3.376	0.103
	Firmicutes	2.808	0.132	11.138	**0.010**	3.658	0.092
	Rokubacteria	3.503	0.098	9.256	**0.016**	4.47	0.067
Fungal phylum	Ascomycota	13.484	**0.006**	0.237	0.640	7.321	**0.027**
	Mortierellomycota	9.767	**0.014**	12.089	**0.008**	12.866	**0.007**
	Basidiomycota	0.019	0.894	5.836	**0.042**	0.192	0.673
	Chytridiomycota	1.234	0.299	1.320	0.284	1.048	0.336
	Glomeromycota	0.095	0.765	2.414	0.159	0.386	0.552
	Mucoromycota	0.001	0.971	5.258	0.051	0.002	0.966
	Kickxellomycota	0.829	0.389	0.829	0.389	1.344	0.280
	Olpidiomycota	0.787	0.401	1.036	0.339	0.959	0.356

The abundance of bacteria was not significantly affected by N addition (Fig. 2A). However, the abundance of fungi increased significantly following N addition (Fig. 2B). There was no significant difference in the abundance of fungi between the N treatment and the N + P treatment ($p > 0.05$). The abundance of both bacteria and fungi was not significantly affected by P addition($p > 0.05$).

Effect of N and P addition on the function of the microbial community

The function of the bacterial community was affected by N and P addition according to FAPROTAX (Fig. 3A). The functions of the bacterial community related to carbon (C) cycling (photoautotrophy, phototrophy, fermentation and chemoheterotrophy) and N cycling (aerobic ammonia oxidation, nitrification) were enhanced in the N treatment. However, the nitrate reduction and predatory or exoparasitic functions decreased following N addition. Nitrification and aerobic ammonia oxidation increased in the P treatment. Chemoheterotrophy, aerobic chemoheterotrophy and aromatic compound degradation increased in the N + P treatment.

The trophic function of the fungal community was affected by N and P addition (Fig. 3B). Pathotroph, saprotroph and pathotroph-saprotroph-symbiotroph groups increased in the N and N + P treatments. The saprotroph group decreased in the P treatment. The symbiotroph group decreased in the N and P treatment. The pathotroph-saprotroph group increased in the N + P treatment. The pathotroph-symbiotroph group enhanced in the P treatment.

Influential Factors On Microbial Community

Results from the SEM showed that N addition explained 98% of the variation in plant biomass, 65% of the variation in soil N:P, 48% of the variation in fungal community, 30% of the variation in bacterial community, and 98% of the variation in activity phosphatase (Fig. 4A). P addition explained 37% of the variation in plant biomass, 88% of the variation in soil N:P, 50% of the variation in fungal community, 45% of the variation in bacterial community, 99% of the variation in activity of urease(Fig. 4B).

N addition significantly increased the plant biomass and soil N:P (Fig. 4A). The plant biomass and fungal community significantly positively affected activity of phosphatase (Fig. 4A). Soil N:P significantly positively affected bacterial community, but did not significantly affected fungal community (Fig. 4A). Soil N:P did not significantly affected activity of phosphatase (Fig. 4A). Fungal community significantly positively affected activity of phosphatase (Fig. 4A). Soil N:P and bacterial community did not significantly affected activity of phosphatase (Fig. 4A).

P addition significantly decreased the soil N:P and did not significantly affected the plant biomass (Fig. 4B). The plant biomass did not significantly affected fungal and bacterial community, but significantly positively affected activity of urease (Fig. 4B). Soil N:P significantly positively affected bacterial community, but did not significantly affected fungal community(Fig. 4B). Soil N:P negatively affected activity of urease (Fig. 4B). Fungal and bacterial community significantly positively affected activity of urease (Fig. 4B).

Discussion

N limitation on the plant shoot biomass and P is synergistic with N in contributing to the biomass

Nitrogen limits biomass in the alpine steppe ecosystems from the analyzing the response ratio of shoot biomass to N and P addition, the results of SEM analysis and field investigation (Table 1, Fig. 4, Table S1). Our results are similar to those of LeBauer and Treseder (2008) and Fay et al. (2015) in that N was found to be the main nutrient factor limiting the biomass of the grassland ecosystem. However, the result from analyzing response ratio of leaf N and P concentration showed that the shoot biomass was co-limited by N and P (Saito et al. 2008; Bracken et al. 2015). Moreover, we found there was no significant difference in the response ratio of shoot biomass and leaf N concentration between the N and the N + P treatments; however, they were higher than that in P treatment (Table 2). Only amendment with P did not increase the shoot biomass (Table S1), which is consistent with the conclusions from other studies also conducted on the grasslands of the Tibetan Plateau (Wang et al. 2015; Dong et al. 2016; Gao et al. 2016). According to response ratio of shoot biomass (LeBauer and Treseder 2008; Fay et al. 2015; Bracken et al. 2015) and the definitions of co-limited or synergistic effects from Sperfeld et al. (2012) and Schleuss et al. (2020), our results suggest that N limits plant biomass of alpine steppe and P is synergistic with N in contributing to plant biomass.

Soil microbes influence the effect of N and P on biomass at alpine grassland

Plants and microbes tend to increase their acquisition of the most limiting nutrient in order to maintain stoichiometric homeostasis (Cleveland and Liptzin 2007; Finkel et al. 2019). Plant growth was activated due to N addition, which maybe induce P deficit (Li et al., 2015). In present study, P content in plant leaf and the soil available phosphate content in the N treatment were the same as those in the CK treatment (Table 3, Table S2), which indicated the deficit of P due to N addition in the N treatment was alleviated. However, Soil inorganic P was strongly bounded by soil minerals and thereby unavailable for plant (Samadi and Gilkes 1999; De Schrijver et al. 2012; Feng et al. 2016). Moreover, organic P mineralization was controlled by soil phosphatase activity (Wang et al. 2016; Margalef et al. 2017). In present study, the activity of neutral and acid phosphatase were promoted in N treatment (Table 3), which suggested that available P in soil will increase due to N addition.

Available P was enhanced by soil microbes due to N addition. When available N resource in soil was increased, plants could provide more carbon substrate for soil microbes, which will accelerate microbial turnover (Ding et al.,2021). From results of SEM, Plant biomass also positively affected fungal community, and then indirectly significantly positively affected phosphatase (Fig. 4A). The phosphatase activity and abundance of fungi were significantly increased by N addition (Table 3, Fig. 2B), which suggests that fungal community had been promoted due to N addition (Hiruma et al. 2016; Almario et al. 2017; Fabiańska et al. 2019). Increasing in root biomass have been shown to increase root exudation and organic substrate, especially in nutrient-poor sites (Bradford et al. 2008; Liu and Greaver 2010), which might change the function of the fungal community because fungal community tend to have a higher use efficiency (Six et al., 2008; Poirier et al., 2018). Shifting symbiotrophic fungi to saprotrophic fungi will

increase the activity of phosphatase (Fig. 3B), which promotes mineralization of soil organic phosphorus (Talbot et al. 2015; Morrison et al. 2016; Li et al. 2019). The relative abundance of ascomycota increased significantly in N treatment (Fig. 1A), which is consistent with the findings of previous studies (Xu et al. 2017; She et al. 2018; Guo et al. 2019; Liu et al. 2020). Ascomycota are key decomposers in soils (Liu et al. 2020), which could rapidly metabolize rhizodeposited organic matter in the rhizosphere soil (Bastida et al. 2013). These results suggested soil fungi also contribute to enhance activity of phosphatase except for plant increasing activity of phosphatase (Ryan et al. 2014; Deng et al. 2017; Finkel et al. 2019; Li et al. 2019; Dai et al. 2020; Schleuss et al. 2020).

P addition will increase N absorption of plant according to N:P stoichiometry. However, the inorganic N concentration is low in alpine grassland (Zhou 2001), the inorganic N pools in soil was determined how much organic N feeds into and supplies the inorganic N pool (Daly et al. 2021). In present study, N content in leaf and soil AN in the P treatment were the same as those in the CK treatment (Table 3, Table S2), which suggested the N deficit of plant due to P addition was alleviated. Results from SEM showed that the fungi and bacteria both significantly positively affected the urease except for plant biomass (Fig. 4B). The FAPROTAX analysis showed that the function of aerobic ammonia oxidation and nitrification and the activity of urease increased significantly following P addition (Fig. 3A, Table 4).

P availability also may play an important role in nitrification processes via structuring soil microbial communities (Godwin and Cotner 2015; Tang et al. 2016; He & Dijkstra 2015; Wei et al. 2017; Cheng et al. 2018). In present study, the relative abundance of bacterial phyla was significantly affected by P addition (Table 3, Fig. 1A). The relative abundance of thaumarchaeota, which perform the first step of nitrification, ammonia oxidation (Biggs-Weber et al. 2020), increased significantly in the P treatment. Although nitrification rates increased with P addition (He and Dijkstra 2015; Wei et al. 2017; Cheng et al. 2018), N mineralization was limited because of the absence of substrate as the shoot biomass and root biomass did not increase significantly due to P addition.

The mutualism of the plant and fungi was also limited when P was added because fungi supply the plant with P in exchange for organic C (Fabiańska et al. 2019). The pathotroph-symbiotroph group was enhanced and the symbiotroph group was weakened following P addition (Fig. 3B), which suggests that mutualism was weakened. In the present study, P addition changed the relative abundance of fungi (Fig. 1B), but did not increase its abundance (Fig. 2B). The relative abundance of mortierellomycota increased significantly with P addition because they can acquire organic N as an N source through chitinolytic activities (Vadivelan and Venkateswaran 2014; Uehling et al. 2017). Pathotrophic-symbiotrophic fungi were enhanced in the P treatment (Fig. 3B), which contributed to ammonification and may transfer N to the plant in growth-limiting N environments (Nicolás et al. 2019; Zak et al. 2019).

Conclusion

Our results suggested that N is the main limiting factor for the biomass of steppe grassland, and P is synergistic with N in contributing to the biomass. The reason is that N addition increases plant biomass

and then increase fungal abundance. Function and composition of fungal community was also changed by N addition, which will enhanced the activity of phosphatase and then contributed to alleviate the P demands of plant due to N addition. P addition did not increased the plant biomass, but enhanced the N requirement for plant. Plant did not affect the fungal and bacterial community. The activity of urease was enhanced due to P addition, which will alleviate the N deficit of plants due to P addition. Therefore, soil microbes will affected the effect of N limitation on plant biomass, in further research, assessments of the limiting factor for the biomass of grassland should consider the effect of soil microbes on N and P cycling.

Declarations

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Conflict of Interest

The authors declare no competing financial interests.

Author's contributions

Our study only include local scientists. Wu Jianbo conceived the ideas and designed the methodology; Wu Jianbo and Zhao Hui collected and analysed the data; Wu Jianbo, Zhao Hui and Wang Xiaodan led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

References

1. Almario J, Jeena G, Wunder J, Langen G, Zuccaro A, Coupland G, Bucher M (2017) Root-associated fungal microbiota of nonmycorrhizal Arabis alpina and its contribution to plant phosphorus nutrition. Proceedings of the National Academy of Sciences 114: 9403–9412. https://doi.org/10.1073/pnas.1710455114
2. Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10. https://doi.org/10.1111/j.1462-2920.2005.00942.x
3. Bastida F, Hernández T, Albaladejo J, García C (2013) Impact of wheat straw decomposition on successional patterns of soil microbial community structure. Soil Biol Biochem 41:262–275. https://doi.org/10.1016/j.soilbio.2013.04.022
4. Biggs-Weber E, Aigle A, Prosser JI, Gubry-Rangin C (2020) Oxygen preference of deeply-rooted mesophilic thaumarchaeota in forest soil. Soil Biol Biochem 148:107848. https://doi.org/10.1016/j.

soilbio.2020.107848

5. Bracken MES, Hillebrand H, Borer ET, Seabloom EW, Cebrian J, Cleland EE, Elser JJ, Gruner DS, Harpole WS, Ngai JT, Smith JE (2015) Signatures of nutrient limitation and co-limitation: responses of autotroph internal nutrient concentrations to nitrogen and phosphorus additions. Oikos 124:113–121. https://doi.org/10.1111/oik.01215
6. Bradford MA, Fierer N, Jackson RB, Maddox TR, Reynolds JF (2008) Nonlinear root-derived carbon sequestration across a gradient of nitrogen and phosphorous deposition in experimental mesocosms. Glob Change Biol 14:1113–1124. https://doi.org/10.1111/j.1365-2486.2008.01564.x
7. Cheng Y, Wang J, Sun N, Xu M, Zhang J, Cai Z, Wang S (2018) Phosphorus addition enhances gross microbial N cycling in phosphorus-poor soils: a 15 N study from two long-term fertilization experiments. Biol Fertil Soils 54:783–789. https://doi.org/10.1007/s00374-018-1294-5
8. Cleveland CC, Liptzin D (2007) C: N: P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass? Biogeochemistry 85:235–252. https://doi.org/10.1007/s10533-007-9132-0
9. Dai Z, Liu G, Chen H, Chen C, Xu J (2019) Long-term nutrient inputs shift soil microbial functional profiles of phosphorus cycling in diverse agroecosystems. The ISME Journal 14:1–14. https://doi.org/10.1038/s41396-019-0567-9
10. Daly AB, Jilling A, Bowles TM, Buchkowski RW, Frey SD, Kallenbach CM, Keiluweit M, Mooshammer M, Schimel JP, Grandy AS (2021) A holistic framework integrating plant-microbe-mineral regulation of soil bioavailable nitrogen. Biogeochemistry. https://doi.org/10.1007/s10533-021-00793-9
11. De Schrijver A, Vesterdal L, Hansen K, De Frenne P, Augusto L, Achat DL, Staelens J, Baeten L, De Keersmaeker L, De Neve S, Verheyen K (2012) Four decades of post-agricultural forest development have caused major redistributions of soil phosphorus fractions. Oecologia 169(1):221–234. https://doi.org/10.1007/s00442-011-2185-8
12. Deng Q, Hui D, Dennis S, Reddy KC (2017) Responses of terrestrial ecosystem phosphorus cycling to nitrogen addition: A meta-analysis. Glob Ecol Biogeogr 26:713–728. https://doi.org/10.1111/geb.12576 26:713–728.
13. Ding WL, Cong WF, Lambers H (2021) Plant phosphorus-acquisition and –use strategies affect soil carbon cycling. Trends in Ecology Evolution 36(10):899–906. https://doi.org/10.1016/j.tree.2021.06.005
14. Dong J, Cui X, Wang S, Wang F, Pang Z, Xu N, Zhao G, Wang S (2016) Changes in biomass and quality of alpine steppe in response to N & P fertilization in the Tibetan Plateau. PLoS One 11. https://doi.org/10.1371/journal.pone.0156146
15. Elser JJ, Bracken ME, Cleland EE, Gruner DS, Harpole WS, Hillebrand H, Ngai JT, Seabloom EW, Shurin JB, Smith JE (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. Ecol Lett 10:1135–1142. https://doi.org/10.1111/j.1461-0248.2007. 01113.x
16. Fabiańska I, Sosa-Lopez E, Bucher M (2019) The role of nutrient balance in shaping plant root-fungal interactions: facts and speculation. Curr Opin Microbiol 49:90–96.

https://doi.org/10.1016/j.mib.2019.10.004

17. Fay PA, Prober SM, Harpole WS, Knops JM, Bakker JD, Borer ET, Lind EM, MacDougall AS, Seabloom EW, Wragg PD (2015) Grassland productivity limited by multiple nutrients. Nature Plants 1:15080. https://doi.org/10.1038/nplants.2015.80
18. Feng J, Turner BL, Lü X, Chen Z, Kai W, Tian J, Chao W, Luo W, Chen L (2016) Phosphorus transformations along a large-scale climosequence in arid and semiarid grasslands of northern China. Global Biogeochem Cycles 30:1264–1275. https://doi.org/10.1002/2015GB005331
19. Fierer N (2017) Embracing the unknown: disentangling the complexities of the soil microbiome. Nat Rev Microbiol 15:579. https://doi.org/10.1038/nrmicro.2017.87
20. Finkel OM, Salas-González I, Castrillo G, Spaepen S, Law TF, Teixeira PJPL, Jones CD, Dangl JL (2019) The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response. PLoS Biol 17:e3000534. https://doi.org/10.1371/journal.pbio.3000534
21. Gao W, Kou L, Zhang J, Müller C, Wang H, Yang H, Li S (2016b) Enhanced deposition of nitrate alters microbial cycling of N in a subtropical forest soil. Biol Fertil Soils 52:977–986. https://doi.org/10.1016/j.apsoil.2016.04.020
22. Godwin CM, Cotner JB (2015) Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. The ISME Journal 9:2324–2327. https://doi.org/10.1038/ismej.2015.34
23. Guo Q, Yan L, Korpelainen H, Niinemets Ü, Li C (2019) Plant-plant interactions and N fertilization shape soil bacterial and fungal communities. Soil Biol Biochem 128:127–138. https://doi.org/10.1016/j.soilbio.2018.10.018
24. He M, Dijkstra FA (2015) Phosphorus addition enhances loss of nitrogen in a phosphorus-poor soil. Soil Biol Biochem 82:99–106. https://doi.org/10.1007/s00374-016-1142-4
25. He D, Xiang X, He J-S, Wang C, Cao G, Adams J, Chu H (2016) Composition of the soil fungal community is more sensitive to phosphorus than nitrogen addition in the alpine meadow on the Qinghai-Tibetan Plateau. Biol Fertil Soils 52:1059–1072. https://doi.org/10.1007/s00374-016-1142-4
26. Hedges LV, Curtis G (1999) The meta-analysis of response ratios in experimental ecology. Ecology 80:1150–1156. https://doi.org/10.2307/177062
27. Hiruma K, Gerlach N, Sacristán S, Nakano RT, Hacquard S, Kracher B, Neumann U, Ramírez D, Bucher M, O'Connell RJ (2016) Root endophyte Colletotrichum to fieldiae confers plant fitness benefits that are phosphate status dependent. Cell 165:464–474. https://doi.org/10.1016/j.cell.2016.02.028
28. Huang Q, Wang J, Wang C, Wang Q (2019) The 19-years inorganic fertilization increased bacterial diversity and altered bacterial community composition and potential functions in a paddy soil. Appl Soil Ecol 144:60–67. https://doi.org/10.1016/j.apsoil.2019.07.009
29. Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13. https://doi.org/10.1016/j.femsec.2003.11.012

30. Kennedy A (1999) Bacterial diversity in agroecosystems. Agriculture Ecosystem Environment 74:65–76. https://doi.org/10.1016/B978-0-444-50019-9.50007-8
31. LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89:371–379. https://doi.org/10.1890/06-2057.1
32. Li Y, Nie C, Liu Y, Du W, He P (2019) Soil microbial community composition closely associates with specific enzyme activities and soil carbon chemistry in a long-term nitrogen fertilized grassland. Sci Total Environ 654:264–274. https://doi.org/10.1016/j.scitotenv.2018.11.031
33. Li Y, Niu SL, Yu GR (2016) Aggravated phosphorus limitation on biomass production under increasing nitrogen loading: a meta-analysis. Glob Change Biol 22:934–943. https://doi.org/10.1111/gcb.13125
34. Liu C, Gong X, Dang K, Li J, Yang P, Gao X, Deng X, Feng B (2020) Linkages between nutrient ratio and the microbial community in rhizosphere soil following fertilizer management. Environ Res 184:109261. https://doi.org/10.1016/j.envres.2020.109261
35. Liu L, Greaver TL (2010) A global perspective on belowground carbon dynamics under nitrogen enrichment. Ecol Lett 13:819–828. https://doi.org/10.1111/j.1461-0248.2010.01482.x
36. Liu X, Zhang Y, Han W, Tang A, Shen J, Cui Z, Vitousek P, Erisman JW, Goulding K, Christie P (2013) Enhanced nitrogen deposition over China. Nature 494:459–462. https://doi.org/10.1111/j.1461-0248.2010.01482.x
37. Long M, Wu H-H, Smith MD, La Pierre KJ, Lü X-T, Zhang H-Y, Han XG, Yu Q (2016) Nitrogen deposition promotes phosphorus uptake of plants in a semi-arid temperate grassland. Plant Soil 408:475–484. https://doi.org/10.1007/s11104-016-3022-y
38. Louca S, Parfrey LW, Doebeli M (2016) Decoupling function and taxonomy in the global ocean microbiome. Science 353:1272–1277. https://10.1126/science.aaf4507
39. Lu RK (1999) Soil and Agro-chemical Analytical Methods. China Agricultural Science and Technology Press, Beijing
40. Margalef O, Sardans J, Fernández-Martínez M, Molowny-Horas R, Janssens IA, Ciais P, Goll D, Richter A, Obersteiner M, Asensio D (2017) Global patterns of phosphatase activity in natural soils. Sci Rep 7:1337. https://doi.org/10.1038/s41598-017-01418-8
41. Marklein AR, Houlton BZ (2012) Nitrogen inputs accelerate phosphorus cycling rates across a wide variety of terrestrial ecosystems. New Phytol 193:696–704. https://doi.org/10.1111/j.1469-8137.2011.03967.x
42. Morrison EW, Frey SD, Sadowsky JJ, van Diepen LT, Thomas WK, Pringle A (2016) Chronic nitrogen additions fundamentally restructure the soil fungal community in a temperate forest. Fungal Ecology 23:48–57. https://doi.org/10.1016/j.funeco.2016.05.011
43. Nannipieri P, Ascher J, Ceccherini M, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655–670. https://doi.org/10.1046/j.1351-0754.2003.0556.x
44. Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, Schilling JS, Kennedy PG (2016) FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild.

Fungal Ecology 20:241–248. https://doi.org/10.1016/j.funeco.2015.06.006

45. Nicolás C, Martin-Bertelsen T, Floudas D, Bentzer J, Smits M, Johansson T, Troein C, Persson P, Tunlid A (2019) The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. The ISME Journal 13:977–988. https://doi.org/10.1038/s41396-018-0331-6
46. Poirier V, Roumet C, Munson AD (2018) The root of the matter: Linking root traits and soil organic matter stabilization processes. Soil Biol Biochem 120:246–259. https://doi.org/10.1016/j.soilbio.2018.02.016
47. Ryan PR, James RA, Weligama C, Delhaize E, Rattey A, Lewis DC, Bovill WD, McDonald G, Rathjen TM, Wang E, Fettell NA, Richardson AE (2014) Can citrate efflux from roots improve phosphorus uptake by plants? Testing the hypothesis with near-isogenic lines of wheat. Plant Physiol 151:230–242. https://doi.org/10.1111/ppl.12150
48. Samadi A, Gilkes RJ (1999) Phosphorus Transformations and Their Relationships with Calcareous Soil Properties of Southern Western Australia. Soil Sci Soc Am J 63:809–815. https://doi.org/10.2136/sssaj1999.634809x
49. Schleuss PM, Widdig M, Heintz-Buschart A, Kirkman K, Spohn M (2020) Interactions of nitrogen and phosphorus cycling promote P acquisition and explain synergistic plant-growth responses. Ecology 101:e03003. https://doi.org/10.1002/ecy.3003
50. She W, Bai Y, Zhang Y, Qin S, Feng W, Sun Y, Zheng J, Wu B (2018) Resource availability drives responses of soil microbial communities to short-term precipitation and nitrogen addition in a desert shrubland. Front Microbiol 9:186. https://doi.org/10.3389/fmicb.2018.00186
51. Six J, Frey SD, Thiet RK, Batten KM (2006) Bacterial and fungal contributions to carbon sequestration in agroecosystems. Soil Sci Soc Am J 70(2):555–569. https://doi.org/10.2136/sssaj2004.0347
52. Sperfeld E, Martin-Creuzburg D, Wacke A (2012) Multiple resource limitation theory applied to herbivorous consumers: Liebig's minimum rule vs. interactive co-limitation. Ecol Lett 15(2):142–150. https://doi.org/10.1111/j.1461-0248.2011.01719.x
53. Stevens C, Lind E, Hautier Y, Harpole W, Borer E, Hobbie S et al (2016) Anthropogenic nitrogen deposition predicts local grassland primary production worldwide. Ecology 96(6):1459–1465. https://doi.org/10.1890/14-1902.1
54. Talbot JM, Martin F, Kohler A, Henrissat B, Peay KG (2015) Functional guild classification predicts the enzymatic role of fungi in litter and soil biogeochemistry. Soil Biol Biochem 88:441–456. https://doi.org/10.1016/j.soilbio.2015.05.006
55. Tang Y, Zhang X, Li D, Wang H, Chen F, Fu X, Fang X, Sun X, Yu G (2016) Impacts of nitrogen and phosphorus additions on the abundance and community structure of ammonia oxidizers and denitrifying bacteria in Chinese fir plantations. Soil Biology and Biochemistry 103: 284–293. https://doi.org/10.1016/j.soilbio. 2016. 09.001
56. Tian XF, Hu HW, Ding Q, Song MH, Xu XL, Zheng Y, Guo LD (2014) Influence of nitrogen fertilization on soil ammonia oxidizer and denitrifier abundance, microbial biomass, and enzyme activities in an

alpine meadow. Biol Fertil Soils 50:703–713. https://doi.org/10.1007/s00374-013-0889-0

57. Uehling J, Gryganskyi A, Hameed K, Tschaplinski T, Misztal P, Wu S, Desirò A, Vande Pol N, Du Z, Zienkiewicz A (2017) Comparative genomics of Mortierella elongata and its bacterial endosymbiont Mycoavidus cysteinexigens. Environ Microbiol 19:2964–2983. https://doi.org/10.1111/1462-2920.13669
58. Vadivelan G, Venkateswaran G (2014) Production and enhancement of omega-3 fatty acid from Mortierella alpina CFR-GV15: its food and therapeutic application. BioMed research international, 2014. https://doi.org/10.1155/2014/657414
59. Wang C, Liu D, Bai E (2018) Decreasing soil microbial diversity is associated with decreasing microbial biomass under nitrogen addition. Soil Biol Biochem 120:126–133. https://doi.org/10.1016/j.soilbio.2018.02.003
60. Wang J, Wang Z, Zhang X, Zhang Y, Ran C, Zhang J, Chen B, Zhang B (2015) Response of Kobresia pygmaea and Stipa purpurea grassland communities in northern Tibet to nitrogen and phosphate addition. Mt Res Dev 35:78–86. https://doi.org/10.1659/MRD-JOURNAL-D-11-00104.1
61. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. https://doi.org/ 10.1128/AEM.00062 – 07
62. Wang R, Creamer CA, Wang X, He P, Xu Z, Jiang Y (2016) The effects of a 9-year nitrogen and water addition on soil aggregate phosphorus and sulfur availability in a semi-arid grassland. Ecologic Indicators 61:806–814. https://doi.org/10.1016/j.ecolind.2015.10.033
63. Wang W, Ma Y, Xu J, Wang H, Zhu J, Zhou H (2012) The uptake diversity of soil nitrogen nutrients by main plant species in Kobresia humilis alpine meadow on the Qinghai-Tibet Plateau. Science China Earth Sciences 55:1688–1695. https://doi.org/10.1007/s11430-012-4461-9
64. Wei X, Hu Y, Peng P, Zhu Z, Atere CT, O'donnell A G, Wu J, Ge T (2017) Effect of P stoichiometry on the abundance of nitrogen-cycle genes in phosphorus-limited paddy soil. Biol Fertil Soils 53:767–776. https://doi.org/10.1007/s00374-017-1221-1
65. Xiang X, Gibbons SM, He J-S, Wang C, He D, Li Q, Ni Y, Chu H (2016) Rapid response of arbuscular mycorrhizal fungal communities to short-term fertilization in an alpine grassland on the Qinghai-Tibet Plateau. Peer Journal 4:e2226. https://doi.org/10.7717/peerj.2226
66. Xu F, Cai T, Yang X, Sui W (2017) Soil fungal community variation by large-scale reclamation in Sanjiang plain, China. Ann Microbiol 67:679–689. https://doi.org/10.1007/s13213-017-1296-9
67. Xu X, Ouyang H, Richter A, Wanek W, Cao G, Kuzyakov Y (2011) Spatio-temporal variations determine plant–microbe competition for inorganic nitrogen in an alpine meadow. Journal Ecology 99:563–571. https://doi.org/10.1111/j.1365-2745.2010.01789.x
68. Zak DR, Pellitier PT, Argiroff W, Castillo B, James TY, Nave LE, Averill C, Beidler KV, Bhatnagar J, Blesh J (2019) Exploring the role of ectomycorrhizal fungi in soil carbon dynamics. New Phytol 223:33–39. https://doi.org/10.1111/nph.15679
69. Zhou XM (2001) Alpine Kobresia Meadows in China. Science Press, Beijing

Figures

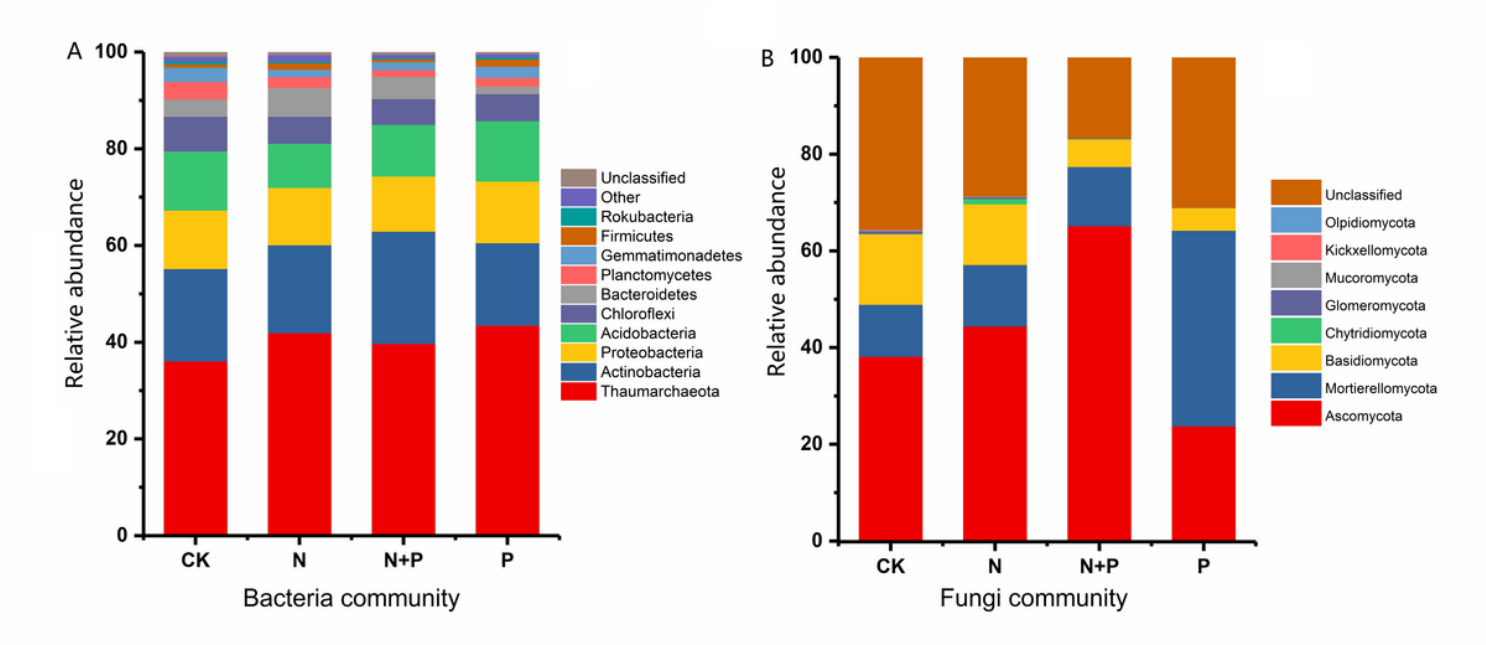

Figure 1

Variation in soil microbial community compositions between different treatments: relative abundance of bacterial phyla (A), relative abundance of fungal phyla (B). Different colors indicate different phyla.

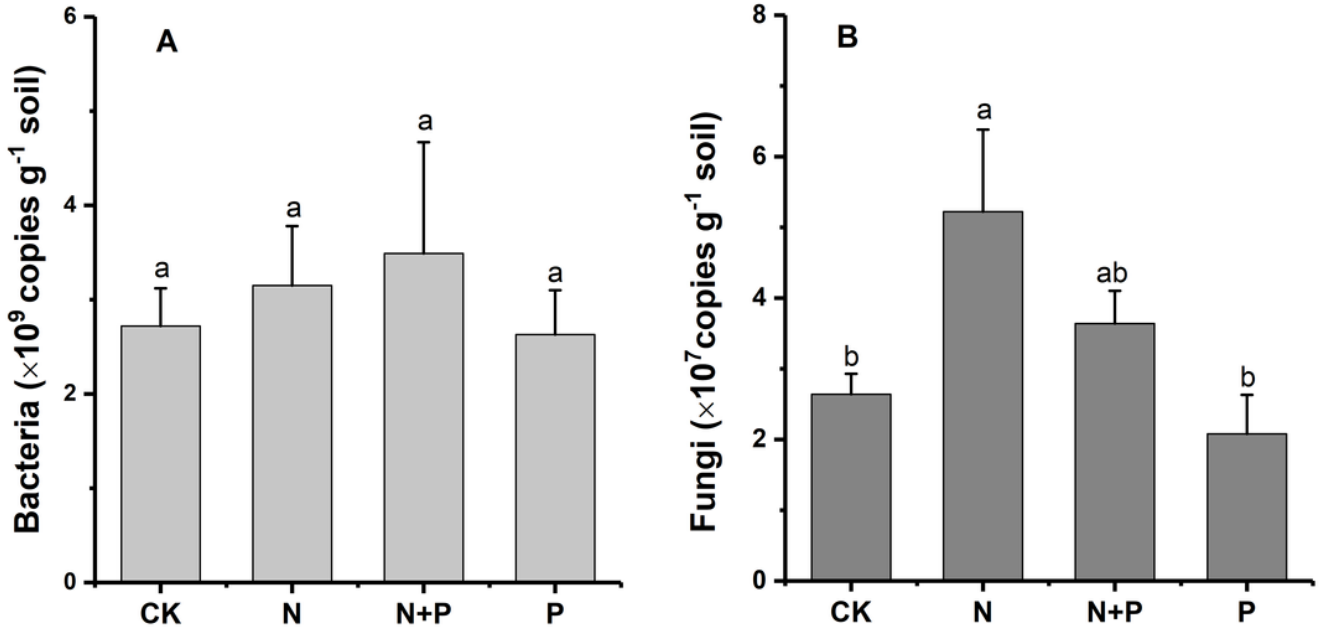

Figure 2

The abundance of bacteria (A) and fungi (B) in the different treatments

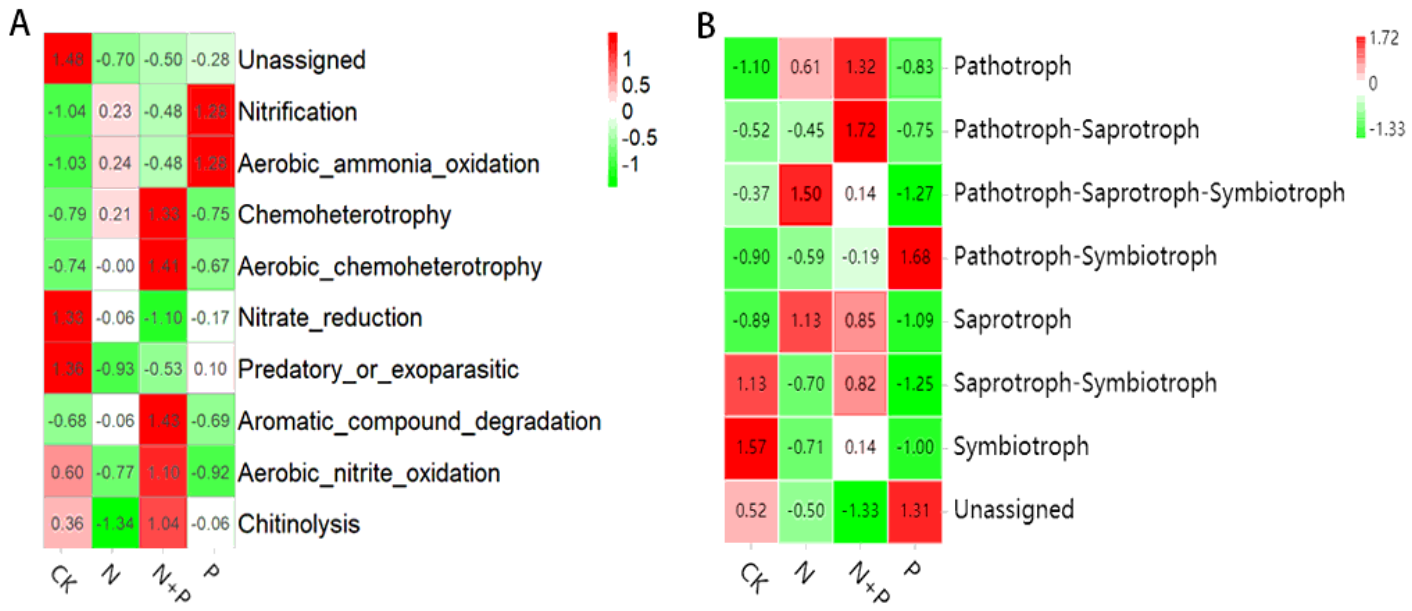

Figure 3

Heatmap representing major differences in predicted functions among different treatments for the functional categories of bacterial communities as predicted by FAPROTAX (A) and the trophic functional categories of fungal communities as predicted by FUNGUILD (B). The numeric values represent relative abundance after z-score in arbitrary units in a row.

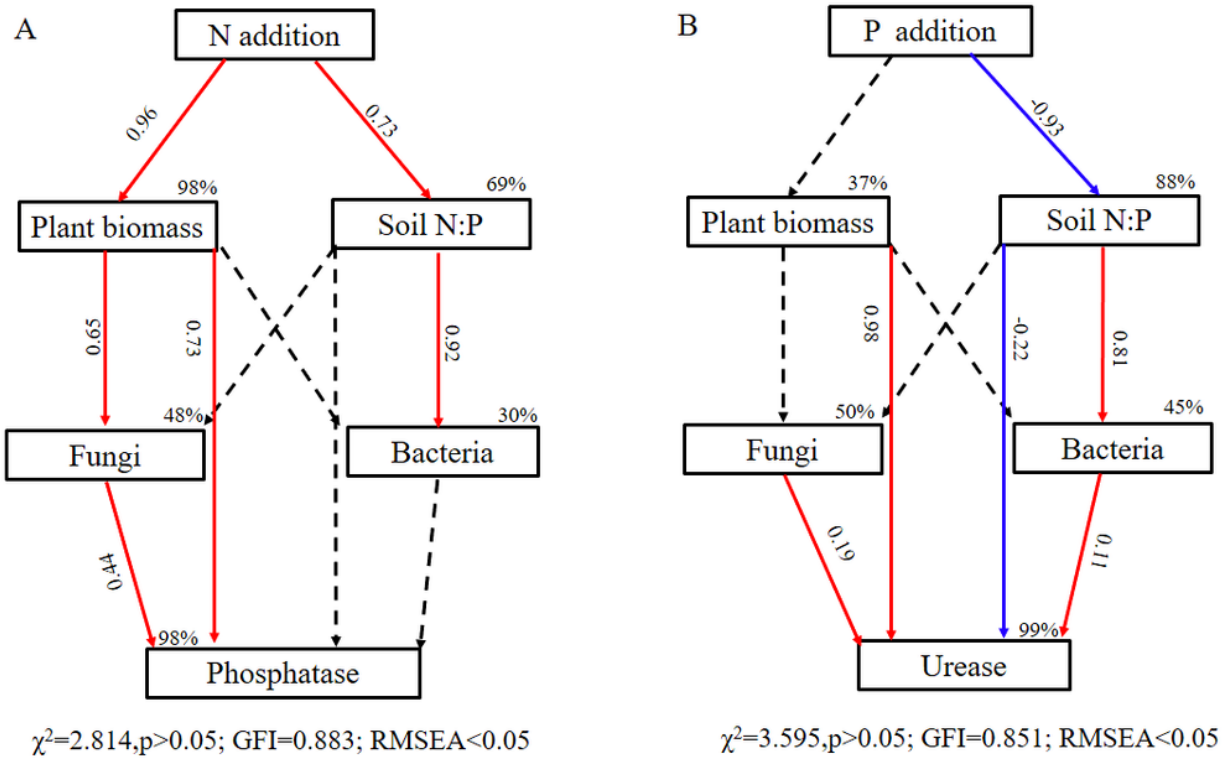

Figure 4

Structural equation model (SEM) explained how the N and P inputs influenced the plant biomass, soil N:P, microbial community and activity of soil enzyme. Red and blue solid arrows represent the positive and negative significant relationships between different variables. Black dash arrows represent the nonsignificant relationships between different variables. The percentage values close to the variables indicate the percentage of the variation in those variables accounted for by the model. Adjacent values near the arrows indicate path coefficients.

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